

PROLIFERATION AND APOPTOSIS RELATED PROTEINS

TECHNICAL FIELD

This invention relates to nucleic acid and amino acid sequences of proliferation and apoptosis related proteins and to the use of these sequences in the diagnosis, treatment, and prevention of cell proliferative, immunological, and reproductive disorders.

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BACKGROUND OF THE INVENTION

Tissue growth involves complex and ordered patterns of cell proliferation, cell differentiation, and regulated cell death (apoptosis). Cell proliferation and apoptosis are regulated to maintain both the number and the spatial organization of cells. This regulation depends on appropriate expression of proteins which control cell cycle progression in response to extracellular signals, such as growth factors and other mitogens, and intracellular cues, such as DNA damage or nutrient starvation. Molecules which directly or indirectly modulate cell cycle progression fall into several categories, including growth factors and their receptors, second messenger and signal transduction proteins, oncogene products, tumor-suppressor proteins, and mitosis-promoting factors. Cancers are characterized by continuous or uncontrolled cell proliferation. Some cancers are associated with suppression of normal apoptotic cell death.

Growth Factors and Signal Transduction Machinery

Growth factors are typically large, secreted polypeptides that act on cells in their local environment to promote cell proliferation. Growth factors bind to and activate specific cell surface receptors that initiate intracellular signal transduction cascades. Many growth factor receptors are classified as receptor tyrosine kinases that undergo autophosphorylation upon ligand binding. Autophosphorylation enables the receptor to interact with signal transduction proteins such as SH2 or SH3 (Src homology regions 2 or 3) domain-containing proteins. Other proteins that act downstream of growth factor receptors contain unique signaling domains such as the SPRY (Sp1a and ryanodine receptor) domain. (See, for example, Schultz, J. et al. (1998) Proc. Natl. Acad. Sci. USA 95:5857-5864.) These proteins then modulate the activity state of small G-proteins, such as Ras, Rab, and Rho, along with GTPase activating proteins (GAPs), guanine nucleotide releasing proteins (GNRPs), and other guanine nucleotide exchange factors. Small G proteins act as molecular switches that turn on mitogen-activated protein kinase (MAP kinase) cascades. MAP kinase activates transcription of the early-response genes discussed below.

Most growth factors also have a multitude of other actions besides the regulation of cell growth and division: they can control the proliferation, survival, differentiation, migration, or function

of cells depending on the circumstance. For example, epidermal growth factor (EGF) protects gastric mucosa against injury and accelerates ulcer healing by stimulating cell migration and proliferation. EGF binds the transmembrane protein tyrosine kinase EGF-R to trigger a series of events that results in activation of the Ras/Raf/MAP kinase pathway by the GTP-binding protein Ras. Other pathways potentially activated by EGF include the phosphatidylinositol pathway and the JAK/STAT signaling pathway (Tarnawski, A.S. et al. (1998) *J. Clin. Gastroenterol.* 27:S12-S20).

In addition to growth factors, small signaling peptides and hormones also influence cell proliferation. These molecules bind primarily to another class of receptor, the trimeric G-protein coupled receptor (GPCR), found predominantly on the surface of immune, neuronal, and neuroendocrine cells. Upon ligand binding, the GPCR activates a trimeric G protein which in turn triggers increased levels of intracellular second messengers such as phospholipase C, Ca^{2+} , and cyclic AMP. Most GPCR-mediated signaling pathways indirectly promote cell proliferation by causing the secretion or breakdown of other signaling molecules that have direct mitogenic effects (Smith, A. et al. (1994) *Cell* 76:959-962).

Protein kinase C (PKC) plays a central role in the control of proliferation and differentiation of various cell types by mediating the signal transduction response to hormones and growth factors. The PKC family of serine/threonine kinases includes twelve different isoforms which have similar catalytic domains at their C-termini, but differ in their N-terminal regulatory domains. Since most cells express multiple PKC isoforms, the specificity of each enzyme for its substrate is achieved by targeting individual isoenzymes to a select location in the cell, either constitutively or upon cell stimulation. A variety of PKC-binding proteins and lipids have been identified that may function to compartmentalize PKC isoenzymes, including RACK1, serum deprivation response (sdr) protein, and SRBC (sdr-related gene product that binds C-kinase). Interestingly, both sdr and SRBC appear to provide localization of activated PKC to caveolae, but each has specificity for a different isoenzyme; sdr interacts specifically with PKC α and SRBC interacts with PKC δ . Both sdr and SRBC are induced during stages of growth arrest, and were originally isolated from serum-deprived cultured cells. Thus, sdr and SRBC appear to be important for targeting activated PKC isoenzymes to subcellular signaling sites important in growth control. (Mineo, C. et al. (1998) *J. Cell Biol.* 141:601-610; and Izumi, Y. et al. (1997) *J. Biol. Chem.* 272:7381-7389.)

Oncogenes

Oncogenes (i.e. "cancer-causing genes") are involved in the reception and transduction of growth factor signals and in the modulation of gene expression in response to these signals. For example, stimulation of a cell by growth factor activates two sets of genes, the early-response genes and the delayed-response genes. Early-response gene products include myc, fos, and jun, all of

which encode gene regulatory proteins. These regulatory proteins activate the transcription of the delayed-response genes which encode proteins directly involved in cell cycle progression, such as the cyclins and cyclin dependent kinase discussed below. Additional oncogene products which directly regulate gene expression include the Rel transcription factor, the Ret zinc finger protein, and the Tre oncoprotein. (See, for example, Cao, T. et al. (1998) J. Cell Sci. 111:1319-1329; and Nakamura, T. et al. (1992) Oncogene 7:733-741.) Some conserved regions of oncogenes have been identified, such as the C3HC4 RING finger motif. Mutations in the C3HC4 RING finger domain of the Bmi-1 oncoprotein, for example, block lymphoma induction in mice (Hemenway, C.S. (1998) Oncogene 16:2541-2547). Apoptosis inhibition motifs have also been identified, such as the BIR repeat implicated in the activity of the IAP (Inhibitor of Apoptosis) family. Mutations or chromosomal translocations which result in hyperactivation of oncogenes result in uncontrolled cell proliferation.

Tumor Suppressors

Tumor suppressor genes are involved in inhibition of cell proliferation. Mutations which decrease the activity of tumor suppressor genes result in increased cell proliferation. In humans and other mammals, tumor suppressors include the retinoblastoma (Rb) and p53 proteins. Tumor suppressors have also been discovered in lower animals such as Drosophila, in which the Discs-Large (Dlg) and Hyperplastic Discs (Hyd) proteins inhibit hyperplasia of undifferentiated epithelial cells in developing imaginal discs. (See, for example, Mansfield, E. et al. (1994) Dev. Biol. 165:507-526.) The importance of tumor suppressor genes and oncogenes in the development of cancer is demonstrated by the fact that about 75% of colorectal cancers have inactivating mutations in the p53 gene and about 50% have a hyper-activating mutation in a ras family oncogene.

Tumor suppressor genes often act as "gatekeepers" (Kinzler, K.W. and Vogelstein, B. (1996) Cell 87:159-170). Normally, the gatekeeper is responsible for maintaining a balance of cell division, growth arrest, and death. External signals may activate or inactivate the gatekeeper, or alter its location within the cell. In some cases, inactivation of the gatekeeper is necessary for cell proliferation, and activation is necessary for cell growth arrest and differentiation. In other cases, the situation is reversed. Proteins which interact with the gatekeeper modify its activity or intracellular location to provide the appropriate response to external signals at any stage in the cell's development.

An example of a gatekeeper protein is the adenomatous polyposis coli (APC) protein. Though APC is expressed ubiquitously, it appears to function as a gatekeeper in colorectal cells. Mutations in the APC protein are linked to familial and sporadic forms of colon cancer. All of these mutations involve truncations in the APC C-terminus, which serves as a binding site for several proteins, including EB1, RPl, and the tumor suppressor protein Dlg. The interactions between APC and these binding proteins may be important for localizing or regulating APC activity. For example,

EB1 appears to link APC to microtubules, and a defect in chromosome segregation has been implicated as an early event in colorectal tumorigenesis (Berreuta, L. (1998) Proc. Natl. Acad. Sci. USA 95:10596-10601; and Renner, C. et al. (1997) J. Immunol. 159:1276-1283).

Another example of a gatekeeper is the E2F transcription factor, which can function either as a positive regulator of cell cycle progression or as a suppressor of cell proliferation, depending on the tissue. The balance of cell division over growth arrest and differentiation appears to involve proteins which interact with and modulate E2F. These proteins include the Rb tumor suppressor protein and NPDC-1 (neural proliferation, differentiation, and control). Rb acts to repress transcriptional activity of E2F, leading to differentiation or apoptosis in the responding cell. NPDC-1 is a neural specific gene expressed in growth arrested and differentiated cells. The NPDC-1 gene product, npdcf-1, interacts with E2F to down-regulate cell proliferation (Dupont, E. et al. (1998) J. Neurosci. Res. 51:257-267).

Cell Cycle Machinery

The molecular machinery which drives the cell cycle in response to mitogens and growth factors has been extensively studied in model systems such as budding yeast, fission yeast, and the African clawed frog, Xenopus. Essentially, the cell cycle is comprised of four successive phases: G1, S (DNA synthesis), G2, and M (mitosis). Cells which exit the cell cycle enter a quiescent phase called G0. Studies in yeast have shown that exit from S and M phases is driven by the anaphase-promoting complex, an assembly of proteins that degrades cyclins via the ubiquitin-mediated protein degradation pathway. (See, for example, Kominami, K. et al. (1998) EMBO J. 17:5388-5399.) Other non-kinase proteins, such as the Zer1p RNA splicing protein in fission yeast, are important for exit of the cell from G0 and entry into G1 or G2. (See, for example, Urushiyama, S. et al. (1997) Genetics 147:101-115.)

Several cell cycle transitions, including the entry and exit of a cell from mitosis, are dependent upon the activation and inhibition of cyclin-dependent kinases (Cdks). The Cdks are composed of a kinase subunit, Cdk, and an activating subunit, cyclin, in a complex that is subject to many levels of regulation. Cyclins bind and activate cyclin-dependent protein kinases which then phosphorylate and activate selected proteins involved in the mitotic process. The Cdk-cyclin complex is both activated and inhibited by phosphorylation. In addition, the Cdk-cyclin complex is regulated by targeted degradation involving molecules such as CDC4 and CDC53. Other proteins mediate entry into or progression through mitosis. For example, Berry and Gould recently identified a novel, 142 amino acid protein from the yeast S. pombe, termed dmp1p, that is required for proper spindle formation and entry into mitosis, but does not interact with cyclin-type proteins (Berry L.D. and Gould K.L. (1997) J. Cell Biol. 137:1337-1354). Dim1p appears to be evolutionarily conserved,

since a human homolog has recently been described (Larin D., et al. (1997) GI 2565275).

Apoptosis Machinery

Apoptosis is the genetically controlled process by which unneeded or defective cells undergo programmed cell death. Selective elimination of cells is as important for morphogenesis and tissue remodeling as is cell proliferation and differentiation. Lack of apoptosis may result in hyperplasia and other disorders associated with increased cell proliferation. Apoptosis is also a critical component of the immune response. Immune cells such as cytotoxic T-cells and natural killer cells prevent the spread of disease by inducing apoptosis in tumor cells and virus-infected cells. In addition, immune cells that fail to distinguish self molecules from foreign molecules must be eliminated by apoptosis to avoid an autoimmune response.

Apoptotic cells undergo distinct morphological changes. Hallmarks of apoptosis include cell shrinkage, nuclear and cytoplasmic condensation, and alterations in plasma membrane topology. Biochemically, apoptotic cells are characterized by increased intracellular calcium concentration, fragmentation of chromosomal DNA, and expression of novel cell surface components.

The molecular mechanisms of apoptosis are highly conserved, and many of the key protein regulators and effectors of apoptosis have been identified. Apoptosis generally proceeds in response to a signal which is transduced intracellularly and results in altered patterns of gene expression and protein activity. Signaling molecules such as hormones and cytokines are known both to stimulate and to inhibit apoptosis through interactions with cell surface receptors. Transcription factors also play an important role in the onset of apoptosis. A number of downstream effector molecules, particularly proteases such as the cysteine proteases called caspases, have been implicated in the degradation of cellular components and the proteolytic activation of other apoptotic effectors.

The Fas/Apo-1 receptor (FAS) is a member of the tumor necrosis factor-receptor family. Upon binding its ligand (Fas ligand), the membrane-spanning FAS induces apoptosis by recruiting several cytoplasmic proteins that transmit the death signal. Chu et al. isolated one such protein from mice, termed FAS-associated protein factor 1 (FAF1), and demonstrated that expression of FAF1 in L cells potentiated FAS-induced apoptosis (Chu, K. et al. (1995) Proc. Natl. Acad. Sci. USA 92:11894-11898). Subsequently, FAS-associated factors have been isolated from numerous other species, including quail and fly (Frohlich, T., et al. (1998) J. Cell Sci. 111:2353-63; and Lukacsovich, T. et al. (1998) GI 3688609).

Fragmentation of chromosomal DNA is one of the hallmarks of apoptosis. DNA fragmentation factor (DFF) is a protein composed of two subunits, a 40-kDa, caspase-activated nuclease termed DFF40/CAD, and its 45-kDa inhibitor DFF45/ICAD. Two mouse homologs of DFF45/ICAD, termed CIDE-A and CIDE-B, have recently been described (Inohara, N. et al. (1998)

EMBO J. 17:2526-2533). CIDE-A and CIDE-B expression in mammalian cells activated apoptosis, while expression of CIDE-A alone induced DNA fragmentation. In addition, FAS-mediated apoptosis was enhanced by CIDE-A and CIDE-B, further implicating these proteins as effectors that mediate apoptosis.

5 Cancers are characterized by inappropriate cell proliferation, which may be due to uncontrolled cell growth or inadequate apoptosis. Strategies for treatment may involve either reestablishing control over cell cycle progression, or selectively stimulating apoptosis in cancerous cells (Nigg, E.A. (1995) BioEssays 17:471-480).

10 Immunological defenses against cancer include induction of apoptosis in mutant cells by tumor suppressors, and the recognition of tumor antigens by T lymphocytes. Response to mitogenic stresses is frequently controlled at the level of transcription and is coordinated by various transcription factors. The Rel/NF-kappa B family of vertebrate transcription factors, for example, plays a pivotal role in inflammatory and immune responses to radiation. The NF-kappa B family includes p50, p52, RelA, RelB, and cRel and other DNA-binding proteins. The p52 protein induces
15 apoptosis, upregulates transcription factor c-Jun, and activates c-Jun N-terminal kinase 1 (JNK1) (Sun, L. et al. (1998) Gene 208:157-166). Most NF-kappa B proteins form DNA-binding homodimers or heterodimers. Dimerization of many transcription factors is mediated by a conserved sequence known as the bZIP domain, characterised by a basic region followed by a leucine zipper.

20 The discovery of new proliferation and apoptosis related proteins and the polynucleotides encoding them satisfies a need in the art by providing new compositions which are useful in the diagnosis, prevention, and treatment of cell proliferative, immunological, and reproductive disorders.

SUMMARY OF THE INVENTION

25 The invention features substantially purified polypeptides, proliferation and apoptosis related proteins, referred to collectively as "PROAP" and individually as "PROAP-1," "PROAP-2," "PROAP-3," "PROAP-4," "PROAP-5," "PROAP-6," "PROAP-7," "PROAP-8," "PROAP-9," "PROAP-10," "PROAP-11," "PROAP-12," "PROAP-13," "PROAP-14," "PROAP-15," "PROAP-16," "PROAP-17," "PROAP-18," and "PROAP-19." In one aspect, the invention provides a substantially purified polypeptide comprising an amino acid sequence selected from the group
30 consisting of SEQ ID NO:1-19 and fragments thereof. The invention also includes a polypeptide comprising an amino acid sequence that differs by one or more conservative amino acid substitutions from an amino acid sequence selected from the group consisting of SEQ ID NO:1-19.

 The invention further provides a substantially purified variant having at least 90% amino acid identity to at least one of the amino acid sequences selected from the group consisting of SEQ ID

NO:1-19 and fragments thereof. The invention also provides an isolated and purified polynucleotide encoding the polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1-19 and fragments thereof. The invention also includes an isolated and purified polynucleotide variant having at least 90% polynucleotide sequence identity to the polynucleotide encoding the polypeptide comprising an amino acid sequence selected from the group consisting of
5 SEQ ID NO:1-19 and fragments thereof.

Additionally, the invention provides an isolated and purified polynucleotide which hybridizes under stringent conditions to the polynucleotide encoding the polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1-19 and fragments thereof. The
10 invention also provides an isolated and purified polynucleotide having a sequence which is complementary to the polynucleotide encoding the polypeptide comprising the amino acid sequence selected from the group consisting of SEQ ID NO:1-19 and fragments thereof.

The invention also provides a method for detecting a polynucleotide in a sample containing nucleic acids, the method comprising the steps of: (a) hybridizing the complement of the
15 polynucleotide sequence to at least one of the polynucleotides of the sample, thereby forming a hybridization complex; and (b) detecting the hybridization complex, wherein the presence of the hybridization complex correlates with the presence of a polynucleotide in the sample. In one aspect, the method further comprises amplifying the polynucleotide prior to hybridization.

The invention also provides an isolated and purified polynucleotide comprising a
20 polynucleotide sequence selected from the group consisting of SEQ ID NO:20-38 and fragments thereof. The invention further provides an isolated and purified polynucleotide variant having at least 90% polynucleotide sequence identity to the polynucleotide sequence selected from the group consisting of SEQ ID NO:20-38 and fragments thereof. The invention also provides an isolated and purified polynucleotide having a sequence which is complementary to the polynucleotide comprising
25 a polynucleotide sequence selected from the group consisting of SEQ ID NO:20-38 and fragments thereof.

The invention further provides an expression vector containing at least a fragment of the polynucleotide encoding the polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1-19. In another aspect, the expression vector is contained within a host
30 cell.

The invention also provides a method for producing a polypeptide, the method comprising the steps of: (a) culturing the host cell containing an expression vector containing a polynucleotide of the invention under conditions suitable for the expression of the polypeptide; and (b) recovering the polypeptide from the host cell culture.

The invention also provides a pharmaceutical composition comprising a substantially purified polypeptide having the amino acid sequence selected from the group consisting of SEQ ID NO:1-19 and fragments thereof, in conjunction with a suitable pharmaceutical carrier.

5 The invention further includes a purified antibody which binds to a polypeptide selected from the group consisting of SEQ ID NO:1-19 and fragments thereof. The invention also provides a purified agonist and a purified antagonist to the polypeptide.

The invention also provides a method for treating or preventing a disorder associated with decreased expression or activity of PROAP, the method comprising administering to a subject in need of such treatment an effective amount of a pharmaceutical composition comprising a substantially
10 purified polypeptide having the amino acid sequence selected from the group consisting of SEQ ID NO:1-19 and fragments thereof, in conjunction with a suitable pharmaceutical carrier.

The invention also provides a method for treating or preventing a disorder associated with increased expression or activity of PROAP, the method comprising administering to a subject in need of such treatment an effective amount of an antagonist of a polypeptide having an amino acid
15 sequence selected from the group consisting of SEQ ID NO:1-19 and fragments thereof.

BRIEF DESCRIPTION OF THE FIGURES AND TABLES

Figures 1A and 1B show the amino acid sequence alignment between PROAP-1 (Incyte ID number 1342011; SEQ ID NO:1) and mouse npdcf-1 (GI 452276; SEQ ID NO:39).

20 Figures 2A and 2B show the amino acid sequence alignment between PROAP-2 (Incyte ID number 1880041; SEQ ID NO:2) and human EB1 (GI 998357; SEQ ID NO:40).

Figure 3 shows the amino acid sequence alignment between PROAP-3 (Incyte ID number 3201881; SEQ ID NO:3) and mouse serum deprivation response (sdr) protein (GI 455719; SEQ ID NO:41).

25 Figure 4 shows the amino acid sequence alignment between PROAP-13 (Incyte ID number 1438978; SEQ ID NO:13) and human dim1p homolog (GI 2565275; SEQ ID NO:42).

Figures 5A and 5B show the amino acid sequence alignment between PROAP-14 (Incyte ID number 2024773; SEQ ID NO:14) and FAS-associated factor from Drosophila melanogaster (GI 3688609; SEQ ID NO:43).

30 Figure 6 shows the amino acid sequence alignment between PROAP-15 (Incyte ID number 3869790; SEQ ID NO:15) and cell death activator CIDE-B from Mus musculus (GI 3114594; SEQ ID NO:44).

The above alignments were produced using the multisequence alignment program of LASERGENE software (DNASTAR, Madison WI).

35 Table 1 shows polypeptide and nucleotide sequence identification numbers (SEQ ID NOs),

clone identification numbers (clone IDs), cDNA libraries, and cDNA fragments used to assemble full-length sequences encoding PROAP.

Table 2 shows features of each polypeptide sequence, including potential motifs, homologous sequences, and methods and algorithms used for identification of PROAP.

5 Table 3 shows selected fragments of each nucleic acid sequence; the tissue-specific expression patterns of each nucleic acid sequence as determined by northern analysis; diseases, disorders, or conditions associated with these tissues; and the vector into which each cDNA was cloned.

10 Table 4 describes the tissues used to construct the cDNA libraries from which cDNA clones encoding PROAP were isolated.

Table 5 shows the tools, programs, and algorithms used to analyze PROAP, along with applicable descriptions, references, and threshold parameters.

DESCRIPTION OF THE INVENTION

15 Before the present proteins, nucleotide sequences, and methods are described, it is understood that this invention is not limited to the particular machines, materials and methods described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

20 It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality of such host cells, and a reference to "an antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

25 Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any machines, materials, and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred machines, materials and methods are now described. All publications mentioned herein are cited for the purpose of describing and disclosing
30 the cell lines, protocols, reagents and vectors which are reported in the publications and which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

DEFINITIONS

"PROAP" refers to the amino acid sequences of substantially purified PROAP obtained from

any species, particularly a mammalian species, including bovine, ovine, porcine, murine, equine, and human, and from any source, whether natural, synthetic, semi-synthetic, or recombinant.

The term "agonist" refers to a molecule which intensifies or mimics the biological activity of PROAP. Agonists may include proteins, nucleic acids, carbohydrates, small molecules, or any other
5 compound or composition which modulates the activity of PROAP either by directly interacting with PROAP or by acting on components of the biological pathway in which PROAP participates.

An "allelic variant" is an alternative form of the gene encoding PROAP. Allelic variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in polypeptides whose structure or function may or may not be altered. A gene may have none, one, or
10 many allelic variants of its naturally occurring form. Common mutational changes which give rise to allelic variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

"Altered" nucleic acid sequences encoding PROAP include those sequences with deletions,
15 insertions, or substitutions of different nucleotides, resulting in a polypeptide the same as PROAP or a polypeptide with at least one functional characteristic of PROAP. Included within this definition are polymorphisms which may or may not be readily detectable using a particular oligonucleotide probe of the polynucleotide encoding PROAP, and improper or unexpected hybridization to allelic variants, with a locus other than the normal chromosomal locus for the polynucleotide sequence encoding
20 PROAP. The encoded protein may also be "altered," and may contain deletions, insertions, or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent PROAP. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues, as long as the biological or immunological activity of PROAP is retained. For example,
25 negatively charged amino acids may include aspartic acid and glutamic acid, and positively charged amino acids may include lysine and arginine. Amino acids with uncharged polar side chains having similar hydrophilicity values may include: asparagine and glutamine; and serine and threonine. Amino acids with uncharged side chains having similar hydrophilicity values may include: leucine, isoleucine, and valine; glycine and alanine; and phenylalanine and tyrosine.

30 The terms "amino acid" and "amino acid sequence" refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these, and to naturally occurring or synthetic molecules. Where "amino acid sequence" is recited to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms are not meant to limit the amino acid sequence to the complete native amino acid sequence associated with the recited protein

molecule.

“Amplification” relates to the production of additional copies of a nucleic acid sequence. Amplification is generally carried out using polymerase chain reaction (PCR) technologies well known in the art.

5 The term “antagonist” refers to a molecule which inhibits or attenuates the biological activity of PROAP. Antagonists may include proteins such as antibodies, nucleic acids, carbohydrates, small molecules, or any other compound or composition which modulates the activity of PROAP either by directly interacting with PROAP or by acting on components of the biological pathway in which PROAP participates.

10 The term “antibody” refers to intact immunoglobulin molecules as well as to fragments thereof, such as Fab, F(ab')₂, and Fv fragments, which are capable of binding an epitopic determinant. Antibodies that bind PROAP polypeptides can be prepared using intact polypeptides or using fragments containing small peptides of interest as the immunizing antigen. The polypeptide or oligopeptide used to immunize an animal (e.g., a mouse, a rat, or a rabbit) can be derived from the
15 translation of RNA, or synthesized chemically, and can be conjugated to a carrier protein if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin (KLH). The coupled peptide is then used to immunize the animal.

The term “antigenic determinant” refers to that region of a molecule (i.e., an epitope) that
20 makes contact with a particular antibody. When a protein or a fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to antigenic determinants (particular regions or three-dimensional structures on the protein). An antigenic determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

25 The term “antisense” refers to any composition containing a nucleic acid sequence which is complementary to the “sense” strand of a specific nucleic acid sequence. Antisense molecules may be produced by any method including synthesis or transcription. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form duplexes and to block either transcription or translation. The designation “negative” or “minus” can refer to
30 the antisense strand, and the designation “positive” or “plus” can refer to the sense strand.

The term “biologically active” refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, “immunologically active” refers to the capability of the natural, recombinant, or synthetic PROAP, or of any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The terms "complementary" and "complementarity" refer to the natural binding of polynucleotides by base pairing. For example, the sequence "5' A-G-T 3'" bonds to the complementary sequence "3' T-C-A 5'." Complementarity between two single-stranded molecules may be "partial," such that only some of the nucleic acids bind, or it may be "complete," such that total complementarity exists between the single stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of the hybridization between the nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between nucleic acid strands, and in the design and use of peptide nucleic acid (PNA) molecules.

A "composition comprising a given polynucleotide sequence" and a "composition comprising a given amino acid sequence" refer broadly to any composition containing the given polynucleotide or amino acid sequence. The composition may comprise a dry formulation or an aqueous solution. Compositions comprising polynucleotide sequences encoding PROAP or fragments of PROAP may be employed as hybridization probes. The probes may be stored in freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. In hybridizations, the probe may be deployed in an aqueous solution containing salts (e.g., NaCl), detergents (e.g., sodium dodecyl sulfate; SDS), and other components (e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.).

"Consensus sequence" refers to a nucleic acid sequence which has been resequenced to resolve uncalled bases, extended using the XL-PCR kit (Perkin-Elmer, Norwalk CT) in the 5' and/or the 3' direction, and resequenced, or which has been assembled from the overlapping sequences of one or more Incyte Clones and, in some cases, one or more public domain ESTs, using a computer program for fragment assembly, such as the GELVIEW fragment assembly system (GCG, Madison WI). Some sequences have been both extended and assembled to produce the consensus sequence.

"Conservative amino acid substitutions" are those substitutions that, when made, least interfere with the properties of the original protein, i.e., the structure and especially the function of the protein is conserved and not significantly changed by such substitutions. The table below shows amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative amino acid substitutions.

Original Residue	Conservative Substitution
Ala	Gly, Ser
Arg	His, Lys
Asn	Asp, Gln, His
Asp	Asn, Glu
Cys	Ala, Ser
Gln	Asn, Glu, His
Glu	Asp, Gln, His
Gly	Ala

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	His	Asn, Arg, Gln, Glu
	Ile	Leu, Val
	Leu	Ile, Val
5	Lys	Arg, Gln, Glu
	Met	Leu, Ile
	Phe	His, Met, Leu, Trp, Tyr
	Ser	Cys, Thr
	Thr	Ser, Val
	Trp	Phe, Tyr
10	Tyr	His, Phe, Trp
	Val	Ile, Leu, Thr

Conservative amino acid substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a beta sheet or alpha helical conformation, (b) the charge or hydrophobicity of the molecule at the site of the substitution, and/or (c) the bulk of the side chain.

A "deletion" refers to a change in the amino acid or nucleotide sequence that results in the absence of one or more amino acid residues or nucleotides.

The term "derivative" refers to the chemical modification of a polypeptide sequence, or a polynucleotide sequence. Chemical modifications of a polynucleotide sequence can include, for example, replacement of hydrogen by an alkyl, acyl, hydroxyl, or amino group. A derivative polynucleotide encodes a polypeptide which retains at least one biological or immunological function of the natural molecule. A derivative polypeptide is one modified by glycosylation, pegylation, or any similar process that retains at least one biological or immunological function of the polypeptide from which it was derived.

A "fragment" is a unique portion of PROAP or the polynucleotide encoding PROAP which is identical in sequence to but shorter in length than the parent sequence. A fragment may comprise up to the entire length of the defined sequence, minus one nucleotide/amino acid residue. For example, a fragment may comprise from 5 to 1000 contiguous nucleotides or amino acid residues. A fragment used as a probe, primer, antigen, therapeutic molecule, or for other purposes, may be at least 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 100, 150, 250 or at least 500 contiguous nucleotides or amino acid residues in length. Fragments may be preferentially selected from certain regions of a molecule. For example, a polypeptide fragment may comprise a certain length of contiguous amino acids selected from the first 250 or 500 amino acids (or first 25% or 50% of a polypeptide) as shown in a certain defined sequence. Clearly these lengths are exemplary, and any length that is supported by the specification, including the Sequence Listing, tables, and figures, may be encompassed by the present embodiments.

A fragment of SEQ ID NO:20-38 comprises a region of unique polynucleotide sequence that

specifically identifies SEQ ID NO:20-38, for example, as distinct from any other sequence in the same genome. A fragment of SEQ ID NO:20-38 is useful, for example, in hybridization and amplification technologies and in analogous methods that distinguish SEQ ID NO:20-38 from related polynucleotide sequences. The precise length of a fragment of SEQ ID NO:20-38 and the region of SEQ ID NO:20-38 to which the fragment corresponds are routinely determinable by one of ordinary skill in the art based on the intended purpose for the fragment.

A fragment of SEQ ID NO:1-19 is encoded by a fragment of SEQ ID NO:20-38. A fragment of SEQ ID NO:1-19 comprises a region of unique amino acid sequence that specifically identifies SEQ ID NO:1-19. For example, a fragment of SEQ ID NO:1-19 is useful as an immunogenic peptide for the development of antibodies that specifically recognize SEQ ID NO:1-19. The precise length of a fragment of SEQ ID NO:1-19 and the region of SEQ ID NO:1-19 to which the fragment corresponds are routinely determinable by one of ordinary skill in the art based on the intended purpose for the fragment.

The term "similarity" refers to a degree of complementarity. There may be partial similarity or complete similarity. The word "identity" may substitute for the word "similarity." A partially complementary sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid is referred to as "substantially similar." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization, and the like) under conditions of reduced stringency. A substantially similar sequence or hybridization probe will compete for and inhibit the binding of a completely similar (identical) sequence to the target sequence under conditions of reduced stringency. This is not to say that conditions of reduced stringency are such that non-specific binding is permitted, as reduced stringency conditions require that the binding of two sequences to one another be a specific (i.e., a selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% similarity or identity). In the absence of non-specific binding, the substantially similar sequence or probe will not hybridize to the second non-complementary target sequence.

The phrases "percent identity" and "% identity," as applied to polynucleotide sequences, refer to the percentage of residue matches between at least two polynucleotide sequences aligned using a standardized algorithm. Such an algorithm may insert, in a standardized and reproducible way, gaps in the sequences being compared in order to optimize alignment between two sequences, and therefore achieve a more meaningful comparison of the two sequences.

Percent identity between polynucleotide sequences may be determined using the default

parameters of the CLUSTAL V algorithm as incorporated into the MEGALIGN version 3.12e sequence alignment program. This program is part of the LASERGENE software package, a suite of molecular biological analysis programs (DNASTAR, Madison WI). CLUSTAL V is described in Higgins, D.G. and P.M. Sharp (1989) CABIOS 5:151-153 and in Higgins, D.G. et al. (1992) CABIOS 8:189-191. For pairwise alignments of polynucleotide sequences, the default parameters are set as follows: Ktuple=2, gap penalty=5, window=4, and "diagonals saved"=4. The "weighted" residue weight table is selected as the default. Percent identity is reported by CLUSTAL V as the "percent similarity" between aligned polynucleotide sequence pairs.

Alternatively, a suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S.F. et al. (1990) J. Mol. Biol. 215:403-410), which is available from several sources, including the NCBI, Bethesda, MD, and on the Internet at <http://www.ncbi.nlm.nih.gov/BLAST/>. The BLAST software suite includes various sequence analysis programs including "blastn," that is used to align a known polynucleotide sequence with other polynucleotide sequences from a variety of databases. Also available is a tool called "BLAST 2 Sequences" that is used for direct pairwise comparison of two nucleotide sequences. "BLAST 2 Sequences" can be accessed and used interactively at <http://www.ncbi.nlm.nih.gov/gorf/bl2.html>. The "BLAST 2 Sequences" tool can be used for both blastn and blastp (discussed below). BLAST programs are commonly used with gap and other parameters set to default settings. For example, to compare two nucleotide sequences, one may use blastn with the "BLAST 2 Sequences" tool Version 2.0.9 (May-07-1999) set at default parameters. Such default parameters may be, for example:

Matrix: BLOSUM62

Reward for match: 1

Penalty for mismatch: -2

Open Gap: 5 and Extension Gap: 2 penalties

Gap x drop-off: 50

Expect: 10

Word Size: 11

Filter: on

Percent identity may be measured over the length of an entire defined sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined sequence, for instance, a fragment of at least 20, at least 30, at least 40, at least 50, at least 70, at least 100, or at least 200 contiguous nucleotides. Such lengths are exemplary only, and it is understood that any fragment length

supported by the sequences shown herein, in the tables, figures, or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences due to the degeneracy of the genetic code. It is understood that changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that all encode substantially the same protein.

The phrases “percent identity” and “% identity,” as applied to polypeptide sequences, refer to the percentage of residue matches between at least two polypeptide sequences aligned using a standardized algorithm. Methods of polypeptide sequence alignment are well-known. Some alignment methods take into account conservative amino acid substitutions. Such conservative substitutions, explained in more detail above, generally preserve the hydrophobicity and acidity at the site of substitution, thus preserving the structure (and therefore function) of the polypeptide.

Percent identity between polypeptide sequences may be determined using the default parameters of the CLUSTAL V algorithm as incorporated into the MEGALIGN version 3.12e sequence alignment program (described and referenced above). For pairwise alignments of polypeptide sequences using CLUSTAL V, the default parameters are set as follows: Ktuple=1, gap penalty=3, window=5, and “diagonals saved”=5. The PAM250 matrix is selected as the default residue weight table. As with polynucleotide alignments, the percent identity is reported by CLUSTAL V as the “percent similarity” between aligned polypeptide sequence pairs.

Alternatively the NCBI BLAST software suite may be used. For example, for a pairwise comparison of two polypeptide sequences, one may use the “BLAST 2 Sequences” tool Version 2.0.9 (May-07-1999) with blastp set at default parameters. Such default parameters may be, for example:

Matrix: BLOSUM62

Open Gap: 11 and Extension Gap: 1 penalties

Gap x drop-off: 50

Expect: 10

Word Size: 3

Filter: on

Percent identity may be measured over the length of an entire defined polypeptide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 70 or at least 150 contiguous residues. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures or Sequence Listing, may be

used to describe a length over which percentage identity may be measured.

“Human artificial chromosomes” (HACs) are linear microchromosomes which may contain DNA sequences of about 6 kb to 10 Mb in size, and which contain all of the elements required for stable mitotic chromosome segregation and maintenance.

5 The term “humanized antibody” refers to antibody molecules in which the amino acid sequence in the non-antigen binding regions has been altered so that the antibody more closely resembles a human antibody, and still retains its original binding ability.

10 “Hybridization” refers to the process by which a polynucleotide strand anneals with a complementary strand through base pairing under defined hybridization conditions. Specific hybridization is an indication that two nucleic acid sequences share a high degree of identity. Specific hybridization complexes form under permissive annealing conditions and remain hybridized after the “washing” step(s). The washing step(s) is particularly important in determining the stringency of the hybridization process, with more stringent conditions allowing less non-specific binding, i.e., binding between pairs of nucleic acid strands that are not perfectly matched. Permissive
15 conditions for annealing of nucleic acid sequences are routinely determinable by one of ordinary skill in the art and may be consistent among hybridization experiments, whereas wash conditions may be varied among experiments to achieve the desired stringency, and therefore hybridization specificity. Permissive annealing conditions occur, for example, at 68°C in the presence of about 6 x SSC, about 1% (w/v) SDS, and about 100 µg/ml denatured salmon sperm DNA.

20 Generally, stringency of hybridization is expressed, in part, with reference to the temperature under which the wash step is carried out. Generally, such wash temperatures are selected to be about 5°C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. An equation for calculating T_m and
25 conditions for nucleic acid hybridization are well known and can be found in Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Press, Plainview NY; specifically see volume 2, chapter 9.

30 High stringency conditions for hybridization between polynucleotides of the present invention include wash conditions of 68°C in the presence of about 0.2 x SSC and about 0.1% SDS, for 1 hour. Alternatively, temperatures of about 65°C, 60°C, 55°C, or 42°C may be used. SSC concentration may be varied from about 0.1 to 2 x SSC, with SDS being present at about 0.1%. Typically, blocking reagents are used to block non-specific hybridization. Such blocking reagents include, for instance, denatured salmon sperm DNA at about 100-200 µg/ml. Organic solvent, such as formamide at a concentration of about 35-50% v/v, may also be used under particular

circumstances, such as for RNA:DNA hybridizations. Useful variations on these wash conditions will be readily apparent to those of ordinary skill in the art. Hybridization, particularly under high stringency conditions, may be suggestive of evolutionary similarity between the nucleotides. Such similarity is strongly indicative of a similar role for the nucleotides and their encoded polypeptides.

5 The term "hybridization complex" refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary bases. A hybridization complex may be formed in solution (e.g., C_0t or R_0t analysis) or formed between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate
10 to which cells or their nucleic acids have been fixed).

The words "insertion" and "addition" refer to changes in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively.

"Immune response" can refer to conditions associated with inflammation, trauma, immune disorders, or infectious or genetic disease, etc. These conditions can be characterized by expression
15 of various factors, e.g., cytokines, chemokines, and other signaling molecules, which may affect cellular and systemic defense systems.

The term "microarray" refers to an arrangement of distinct polynucleotides on a substrate.

The terms "element" and "array element" in a microarray context, refer to hybridizable polynucleotides arranged on the surface of a substrate.

20 The term "modulate" refers to a change in the activity of PROAP. For example, modulation may cause an increase or a decrease in protein activity, binding characteristics, or any other biological, functional, or immunological properties of PROAP.

The phrases "nucleic acid" and "nucleic acid sequence" refer to a nucleotide, oligonucleotide, polynucleotide, or any fragment thereof. These phrases also refer to DNA or RNA of genomic or
25 synthetic origin which may be single-stranded or double-stranded and may represent the sense or the antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material.

"Operably linked" refers to the situation in which a first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding
30 sequence. Generally, operably linked DNA sequences may be in close proximity or contiguous and, where necessary to join two protein coding regions, in the same reading frame.

"Peptide nucleic acid" (PNA) refers to an antisense molecule or anti-gene agent which comprises an oligonucleotide of at least about 5 nucleotides in length linked to a peptide backbone of amino acid residues ending in lysine. The terminal lysine confers solubility to the composition.

PNAs preferentially bind complementary single stranded DNA or RNA and stop transcript elongation, and may be pegylated to extend their lifespan in the cell.

"Probe" refers to nucleic acid sequences encoding PROAP, their complements, or fragments thereof, which are used to detect identical, allelic or related nucleic acid sequences. Probes are

isolated oligonucleotides or polynucleotides attached to a detectable label or reporter molecule.

Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes.

"Primers" are short nucleic acids, usually DNA oligonucleotides, which may be annealed to a target polynucleotide by complementary base-pairing. The primer may then be extended along the target

DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification (and

identification) of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR).

Probes and primers as used in the present invention typically comprise at least 15 contiguous nucleotides of a known sequence. In order to enhance specificity, longer probes and primers may

also be employed, such as probes and primers that comprise at least 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or at least 150 consecutive nucleotides of the disclosed nucleic acid sequences. Probes and

primers may be considerably longer than these examples, and it is understood that any length supported by the specification, including the tables, figures, and Sequence Listing, may be used.

Methods for preparing and using probes and primers are described in the references, for example Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Press, Plainview NY; Ausubel et al., 1987, Current Protocols in Molecular Biology,

Greene Publ. Assoc. & Wiley-Intersciences, New York NY; Innis et al., 1990, PCR Protocols, A Guide to Methods and Applications, Academic Press, San Diego CA. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, 1991, Whitehead Institute for Biomedical Research, Cambridge MA).

Oligonucleotides for use as primers are selected using software known in the art for such purpose. For example, OLIGO 4.06 software is useful for the selection of PCR primer pairs of up to 100 nucleotides each, and for the analysis of oligonucleotides and larger polynucleotides of up to 5,000 nucleotides from an input polynucleotide sequence of up to 32 kilobases. Similar primer selection programs have incorporated additional features for expanded capabilities. For example, the PrimOU primer selection program (available to the public from the Genome Center at University of Texas South West Medical Center, Dallas TX) is capable of choosing specific primers from megabase sequences and is thus useful for designing primers on a genome-wide scope. The Primer3 primer selection program (available to the public from the Whitehead Institute/MIT Center for Genome Research, Cambridge MA) allows the user to input a "mispriming library," in which sequences to avoid as primer binding sites are user-specified. Primer3 is useful, in particular, for the

selection of oligonucleotides for microarrays. (The source code for the latter two primer selection programs may also be obtained from their respective sources and modified to meet the user's specific needs.) The PrimeGen program (available to the public from the UK Human Genome Mapping Project Resource Centre, Cambridge UK) designs primers based on multiple sequence alignments, thereby allowing selection of primers that hybridize to either the most conserved or least conserved regions of aligned nucleic acid sequences. Hence, this program is useful for identification of both unique and conserved oligonucleotides and polynucleotide fragments. The oligonucleotides and polynucleotide fragments identified by any of the above selection methods are useful in hybridization technologies, for example, as PCR or sequencing primers, microarray elements, or specific probes to identify fully or partially complementary polynucleotides in a sample of nucleic acids. Methods of oligonucleotide selection are not limited to those described above.

A "recombinant nucleic acid" is a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two or more otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques such as those described in Sambrook, supra. The term recombinant includes nucleic acids that have been altered solely by addition, substitution, or deletion of a portion of the nucleic acid. Frequently, a recombinant nucleic acid may include a nucleic acid sequence operably linked to a promoter sequence. Such a recombinant nucleic acid may be part of a vector that is used, for example, to transform a cell.

Alternatively, such recombinant nucleic acids may be part of a viral vector, e.g., based on a vaccinia virus, that could be used to vaccinate a mammal wherein the recombinant nucleic acid is expressed, inducing a protective immunological response in the mammal.

The term "sample" is used in its broadest sense. A sample suspected of containing nucleic acids encoding PROAP, or fragments thereof, or PROAP itself, may comprise a bodily fluid; an extract from a cell, chromosome, organelle, or membrane isolated from a cell; a cell; genomic DNA, RNA, or cDNA, in solution or bound to a substrate; a tissue; a tissue print; etc.

The terms "specific binding" and "specifically binding" refer to that interaction between a protein or peptide and an agonist, an antibody, an antagonist, a small molecule, or any natural or synthetic binding composition. The interaction is dependent upon the presence of a particular structure of the protein, e.g., the antigenic determinant or epitope, recognized by the binding molecule. For example, if an antibody is specific for epitope "A," the presence of a polypeptide containing the epitope A, or the presence of free unlabeled A, in a reaction containing free labeled A and the antibody will reduce the amount of labeled A that binds to the antibody.

The term "substantially purified" refers to nucleic acid or amino acid sequences that are removed from their natural environment and are isolated or separated, and are at least about 60% free, preferably about 75% free, and most preferably about 90% free from other components with which they are naturally associated.

5 A "substitution" refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

"Substrate" refers to any suitable rigid or semi-rigid support including membranes, filters, chips, slides, wafers, fibers, magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles and capillaries. The substrate can have a variety of surface forms, such as wells,
10 trenches, pins, channels and pores, to which polynucleotides or polypeptides are bound.

"Transformation" describes a process by which exogenous DNA enters and changes a recipient cell. Transformation may occur under natural or artificial conditions according to various methods well known in the art, and may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method for transformation is
15 selected based on the type of host cell being transformed and may include, but is not limited to, viral infection, electroporation, heat shock, lipofection, and particle bombardment. The term "transformed" cells includes stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome, as well as transiently transformed cells which express the inserted DNA or RNA for limited periods of time.

20 A "variant" of a particular nucleic acid sequence is defined as a nucleic acid sequence having at least 40% sequence identity to the particular nucleic acid sequence over a certain length of one of the nucleic acid sequences using blastn with the "BLAST 2 Sequences" tool Version 2.0.9 (May-07-1999) set at default parameters. Such a pair of nucleic acids may show, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% or
25 greater sequence identity over a certain defined length. A variant may be described as, for example, an "allelic" (as defined above), "splice," "species," or "polymorphic" variant. A splice variant may have significant identity to a reference molecule, but will generally have a greater or lesser number of polynucleotides due to alternate splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or lack domains that are present in the
30 reference molecule. Species variants are polynucleotide sequences that vary from one species to another. The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals of a given species. Polymorphic variants also may encompass "single nucleotide polymorphisms" (SNPs) in which the polynucleotide sequence varies by one nucleotide base. The

presence of SNPs may be indicative of, for example, a certain population, a disease state, or a propensity for a disease state.

A "variant" of a particular polypeptide sequence is defined as a polypeptide sequence having at least 40% sequence identity to the particular polypeptide sequence over a certain length of one of the polypeptide sequences using blastp with the "BLAST 2 Sequences" tool Version 2.0.9 (May-07-1999) set at default parameters. Such a pair of polypeptides may show, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% or greater sequence identity over a certain defined length of one of the polypeptides.

10 THE INVENTION

The invention is based on the discovery of new human proliferation and apoptosis related proteins (PROAP), the polynucleotides encoding PROAP, and the use of these compositions for the diagnosis, treatment, or prevention of cell proliferative, immunological, and reproductive disorders.

Table 1 lists the Incyte clones used to assemble full length nucleotide sequences encoding PROAP. Columns 1 and 2 show the sequence identification numbers (SEQ ID NOs) of the polypeptide and nucleotide sequences, respectively. Column 3 shows the clone IDs of the Incyte clones in which nucleic acids encoding each PROAP were identified, and column 4 shows the cDNA libraries from which these clones were isolated. Column 5 shows Incyte clones and their corresponding cDNA libraries. Clones for which cDNA libraries are not indicated were derived from pooled cDNA libraries. The Incyte clones in column 5 were used to assemble the consensus nucleotide sequence of each PROAP and are useful as fragments in hybridization technologies.

The columns of Table 2 show various properties of each of the polypeptides of the invention: column 1 references the SEQ ID NO; column 2 shows the number of amino acid residues in each polypeptide; column 3 shows potential phosphorylation sites; column 4 shows potential glycosylation sites; column 5 shows the amino acid residues comprising signature sequences and motifs; column 6 shows homologous sequences as identified by BLAST analysis; and column 7 shows analytical methods used to identify each polypeptide through sequence homology and protein motifs.

As shown in Figures 1A and 1B, PROAP-1 has chemical and structural similarity with mouse npdcf-1 (GI 452276; SEQ ID NO:39). In particular, PROAP-1 and npdcf-1 share 66% identity and have similar isoelectric points (7.5 and 7.2, respectively). As shown in Figures 2A and 2B, PROAP-2 has chemical and structural similarity with human EB1 (GI 998357; SEQ ID NO:40). In particular, PROAP-2 and EB1 share 64% identity and have similar isoelectric points (5.3 and 4.9, respectively). As shown in Figure 3, PROAP-3 has chemical and structural similarity with mouse serum deprivation response (sdr) protein (GI 455719; SEQ ID NO:41). In particular, PROAP-3 is 86% identical to sdr

from residue M1 through V239 on sdr. As shown in Figure 4, PROAP-13 has chemical and structural similarity with human dim1p homolog (GI 2565275; SEQ ID NO:42). In particular, PROAP-13 and Dim1p share 36% identity. As shown in Figures 5A and 5B, PROAP-14 has chemical and structural similarity with Fly FAS-associated factor (FFAF) from D. melanogaster (GI 3688609; SEQ ID NO:43). In particular, PROAP-14 and FFAF share 40% identity. As shown in Figure 6, PROAP-15 has chemical and structural similarity with cell death activator CIDE-B from M. musculus (GI 3114594; SEQ ID NO:44). In particular, PROAP-15 and CIDE-B share 83% identity.

The columns of Table 3 show the tissue-specificity and diseases, disorders, or conditions associated with nucleotide sequences encoding PROAP. The first column of Table 3 lists the

nucleotide SEQ ID NOs. Column 2 lists fragments of the nucleotide sequences of column 1. These fragments are useful, for example, in hybridization or amplification technologies to identify SEQ ID NO:20-38 and to distinguish between SEQ ID NO:20-38 and related polynucleotide sequences. The polypeptides encoded by these fragments are useful, for example, as immunogenic peptides. Column 3 lists tissue categories which express PROAP as a fraction of total tissues expressing PROAP.

Column 4 lists diseases, disorders, or conditions associated with those tissues expressing PROAP as a fraction of total tissues expressing PROAP. Column 5 lists the vectors used to subclone each cDNA library. Of particular note is the expression of SEQ ID NO:20 in reproductive, nervous, and cardiovascular tissues, of SEQ ID NO:21 in nervous tissue, of SEQ ID NO:22 in reproductive and gastrointestinal tissues, of SEQ ID NO:28, which is detected exclusively in a cDNA library derived from tibia meniscus tissue, of SEQ ID NO:30, which is detected exclusively in a cDNA library derived from diseased liver, of SEQ ID NO:32 in brain tumor-associated tissues, of SEQ ID NO:33 in tumors of the breast and brain, and of SEQ ID NO:34 in tumors of the breast and testicle.

The columns of Table 4 show descriptions of the tissues used to construct the cDNA libraries from which cDNA clones encoding PROAP were isolated. Column 1 references the nucleotide SEQ ID NOs, column 2 shows the cDNA libraries from which these clones were isolated, and column 3 shows the tissue origins and other descriptive information relevant to the cDNA libraries in column 2.

The invention also encompasses PROAP variants. A preferred PROAP variant is one which has at least about 80%, or alternatively at least about 90%, or even at least about 95% amino acid sequence identity to the PROAP amino acid sequence, and which contains at least one functional or structural characteristic of PROAP.

The invention also encompasses polynucleotides which encode PROAP. In a particular embodiment, the invention encompasses a polynucleotide sequence comprising a sequence selected from the group consisting of SEQ ID NO:20-38, which encodes PROAP.

The invention also encompasses a variant of a polynucleotide sequence encoding PROAP. In

particular, such a variant polynucleotide sequence will have at least about 80%, or alternatively at least about 90%, or even at least about 95% polynucleotide sequence identity to the polynucleotide sequence encoding PROAP. A particular aspect of the invention encompasses a variant of a polynucleotide sequence comprising a sequence selected from the group consisting of SEQ ID
5 NO:20-38 which has at least about 80%, or alternatively at least about 90%, or even at least about 95% polynucleotide sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO:20-38. Any one of the polynucleotide variants described above can encode an amino acid sequence which contains at least one functional or structural characteristic of PROAP.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the
10 genetic code, a multitude of polynucleotide sequences encoding PROAP, some bearing minimal similarity to the polynucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention contemplates each and every possible variation of polynucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the
15 polynucleotide sequence of naturally occurring PROAP, and all such variations are to be considered as being specifically disclosed.

Although nucleotide sequences which encode PROAP and its variants are generally capable of hybridizing to the nucleotide sequence of the naturally occurring PROAP under appropriately selected conditions of stringency, it may be advantageous to produce nucleotide sequences encoding
20 PROAP or its derivatives possessing a substantially different codon usage, e.g., inclusion of non-naturally occurring codons. Codons may be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding PROAP and its derivatives without altering the encoded amino acid
25 sequences include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

The invention also encompasses production of DNA sequences which encode PROAP and PROAP derivatives, or fragments thereof, entirely by synthetic chemistry. After production, the synthetic sequence may be inserted into any of the many available expression vectors and cell
30 systems using reagents well known in the art. Moreover, synthetic chemistry may be used to introduce mutations into a sequence encoding PROAP or any fragment thereof.

Also encompassed by the invention are polynucleotide sequences that are capable of hybridizing to the claimed polynucleotide sequences, and, in particular, to those shown in SEQ ID NO:20-38 and fragments thereof under various conditions of stringency. (See, e.g., Wahl, G.M. and

S.L. Berger (1987) *Methods Enzymol.* 152:399-407; Kimmel, A.R. (1987) *Methods Enzymol.* 152:507-511.) Hybridization conditions, including annealing and wash conditions, are described in "Definitions."

Methods for DNA sequencing are well known in the art and may be used to practice any of the embodiments of the invention. The methods may employ such enzymes as the Klenow fragment of DNA polymerase I, SEQUENASE (US Biochemical, Cleveland OH), Taq polymerase (Perkin-Elmer), thermostable T7 polymerase (Amersham Pharmacia Biotech, Piscataway NJ), or combinations of polymerases and proofreading exonucleases such as those found in the ELONGASE amplification system (Life Technologies, Gaithersburg MD). Preferably, sequence preparation is automated with machines such as the MICROLAB 2200 liquid transfer system (Hamilton, Reno NV), PTC200 thermal cycler (MJ Research, Watertown MA) and ABI CATALYST 800 thermal cycler (Perkin-Elmer). Sequencing is then carried out using either the ABI 373 or 377 DNA sequencing system (Perkin-Elmer), the MEGABACE 1000 DNA sequencing system (Molecular Dynamics, Sunnyvale CA), or other systems known in the art. The resulting sequences are analyzed using a variety of algorithms which are well known in the art. (See, e.g., Ausubel, F.M. (1997) Short Protocols in Molecular Biology, John Wiley & Sons, New York NY, unit 7.7; Meyers, R.A. (1995) Molecular Biology and Biotechnology, Wiley VCH, New York NY, pp. 856-853.)

The nucleic acid sequences encoding PROAP may be extended utilizing a partial nucleotide sequence and employing various PCR-based methods known in the art to detect upstream sequences, such as promoters and regulatory elements. For example, one method which may be employed, restriction-site PCR, uses universal and nested primers to amplify unknown sequence from genomic DNA within a cloning vector. (See, e.g., Sarkar, G. (1993) *PCR Methods Applic.* 2:318-322.) Another method, inverse PCR, uses primers that extend in divergent directions to amplify unknown sequence from a circularized template. The template is derived from restriction fragments comprising a known genomic locus and surrounding sequences. (See, e.g., Triglia, T. et al. (1988) *Nucleic Acids Res.* 16:8186.) A third method, capture PCR, involves PCR amplification of DNA fragments adjacent to known sequences in human and yeast artificial chromosome DNA. (See, e.g., Lagerstrom, M. et al. (1991) *PCR Methods Applic.* 1:111-119.) In this method, multiple restriction enzyme digestions and ligations may be used to insert an engineered double-stranded sequence into a region of unknown sequence before performing PCR. Other methods which may be used to retrieve unknown sequences are known in the art. (See, e.g., Parker, J.D. et al. (1991) *Nucleic Acids Res.* 19:3055-3060). Additionally, one may use PCR, nested primers, and PROMOTERFINDER libraries (Clontech, Palo Alto CA) to walk genomic DNA. This procedure avoids the need to screen libraries and is useful in finding intron/exon junctions. For all PCR-based methods, primers may be designed

using commercially available software, such as OLIGO 4.06 Primer Analysis software (National Biosciences, Plymouth MN) or another appropriate program, to be about 22 to 30 nucleotides in length, to have a GC content of about 50% or more, and to anneal to the template at temperatures of about 68°C to 72°C.

5 When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. In addition, random-primed libraries, which often include sequences containing the 5' regions of genes, are preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries may be useful for extension of sequence into 5' non-transcribed regulatory regions.

10 Capillary electrophoresis systems which are commercially available may be used to analyze the size or confirm the nucleotide sequence of sequencing or PCR products. In particular, capillary sequencing may employ flowable polymers for electrophoretic separation, four different nucleotide-specific, laser-stimulated fluorescent dyes, and a charge coupled device camera for detection of the emitted wavelengths. Output/light intensity may be converted to electrical signal using appropriate
15 software (e.g., GENOTYPER and SEQUENCE NAVIGATOR, Perkin-Elmer), and the entire process from loading of samples to computer analysis and electronic data display may be computer controlled. Capillary electrophoresis is especially preferable for sequencing small DNA fragments which may be present in limited amounts in a particular sample.

In another embodiment of the invention, polynucleotide sequences or fragments thereof
20 which encode PROAP may be cloned in recombinant DNA molecules that direct expression of PROAP, or fragments or functional equivalents thereof, in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be produced and used to express PROAP.

The nucleotide sequences of the present invention can be engineered using methods generally
25 known in the art in order to alter PROAP-encoding sequences for a variety of purposes including, but not limited to, modification of the cloning, processing, and/or expression of the gene product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. For example, oligonucleotide-mediated site-directed mutagenesis may be used to introduce mutations that create new restriction
30 sites, alter glycosylation patterns, change codon preference, produce splice variants, and so forth.

In another embodiment, sequences encoding PROAP may be synthesized, in whole or in part, using chemical methods well known in the art. (See, e.g., Caruthers, M.H. et al. (1980) Nucleic Acids Symp. Ser. 7:215-223; and Horn, T. et al. (1980) Nucleic Acids Symp. Ser. 7:225-232.) Alternatively, PROAP itself or a fragment thereof may be synthesized using chemical methods. For

example, peptide synthesis can be performed using various solid-phase techniques. (See, e.g., Roberge, J.Y. et al. (1995) *Science* 269:202-204.) Automated synthesis may be achieved using the ABI 431A peptide synthesizer (Perkin-Elmer). Additionally, the amino acid sequence of PROAP, or any part thereof, may be altered during direct synthesis and/or combined with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

The peptide may be substantially purified by preparative high performance liquid chromatography. (See, e.g., Chiez, R.M. and F.Z. Regnier (1990) *Methods Enzymol.* 182:392-421.) The composition of the synthetic peptides may be confirmed by amino acid analysis or by sequencing. (See, e.g., Creighton, T. (1984) Proteins, Structures and Molecular Properties, WH Freeman, New York NY.)

In order to express a biologically active PROAP, the nucleotide sequences encoding PROAP or derivatives thereof may be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for transcriptional and translational control of the inserted coding sequence in a suitable host. These elements include regulatory sequences, such as enhancers, constitutive and inducible promoters, and 5' and 3' untranslated regions in the vector and in polynucleotide sequences encoding PROAP. Such elements may vary in their strength and specificity. Specific initiation signals may also be used to achieve more efficient translation of sequences encoding PROAP. Such signals include the ATG initiation codon and adjacent sequences, e.g. the Kozak sequence. In cases where sequences encoding PROAP and its initiation codon and upstream regulatory sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals including an in-frame ATG initiation codon should be provided by the vector. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for the particular host cell system used. (See, e.g., Scharf, D. et al. (1994) *Results Probl. Cell Differ.* 20:125-162.)

Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding PROAP and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. (See, e.g., Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview NY, ch. 4, 8, and 16-17; Ausubel, F.M. et al. (1995) Current Protocols in Molecular Biology, John Wiley & Sons, New York NY, ch. 9, 13, and 16.)

A variety of expression vector/host systems may be utilized to contain and express sequences

encoding PROAP. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with viral expression vectors (e.g., baculovirus); plant cell systems transformed with viral expression vectors (e.g., cauliflower mosaic virus, CaMV, or tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems. The invention is not limited by the host cell employed.

In bacterial systems, a number of cloning and expression vectors may be selected depending upon the use intended for polynucleotide sequences encoding PROAP. For example, routine cloning, subcloning, and propagation of polynucleotide sequences encoding PROAP can be achieved using a multifunctional *E. coli* vector such as PBLUESCRIPT (Stratagene, La Jolla CA) or PSORT1 plasmid (Life Technologies). Ligation of sequences encoding PROAP into the vector's multiple cloning site disrupts the *lacZ* gene, allowing a colorimetric screening procedure for identification of transformed bacteria containing recombinant molecules. In addition, these vectors may be useful for *in vitro* transcription, dideoxy sequencing, single strand rescue with helper phage, and creation of nested deletions in the cloned sequence. (See, e.g., Van Heeke, G. and S.M. Schuster (1989) J. Biol. Chem. 264:5503-5509.) When large quantities of PROAP are needed, e.g. for the production of antibodies, vectors which direct high level expression of PROAP may be used. For example, vectors containing the strong, inducible T5 or T7 bacteriophage promoter may be used.

Yeast expression systems may be used for production of PROAP. A number of vectors containing constitutive or inducible promoters, such as alpha factor, alcohol oxidase, and PGH promoters, may be used in the yeast *Saccharomyces cerevisiae* or *Pichia pastoris*. In addition, such vectors direct either the secretion or intracellular retention of expressed proteins and enable integration of foreign sequences into the host genome for stable propagation. (See, e.g., Ausubel, 1995, *supra*; Bitter, G.A. et al. (1987) Methods Enzymol. 153:516-544; and Scorer, C.A. et al. (1994) Bio/Technology 12:181-184.)

Plant systems may also be used for expression of PROAP. Transcription of sequences encoding PROAP may be driven viral promoters, e.g., the 35S and 19S promoters of CaMV used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) EMBO J. 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used. (See, e.g., Coruzzi, G. et al. (1984) EMBO J. 3:1671-1680; Broglie, R. et al. (1984) Science 224:838-843; and Winter, J. et al. (1991) Results Probl. Cell Differ. 17:85-105.) These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. (See, e.g., *The McGraw Hill Yearbook of Science and Technology* (1992) McGraw Hill, New York NY, pp. 191-196.)

In mammalian cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding PROAP may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain
 5 infective virus which expresses PROAP in host cells. (See, e.g., Logan, J. and T. Shenk (1984) Proc. Natl. Acad. Sci. USA 81:3655-3659.) In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells. SV40 or EBV-based vectors may also be used for high-level protein expression.

Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of
 10 DNA than can be contained in and expressed from a plasmid. HACs of about 6 kb to 10 Mb are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes. (See, e.g., Harrington, J.J. et al. (1997) Nat. Genet. 15:345-355.)

For long term production of recombinant proteins in mammalian systems, stable expression
 15 of PROAP in cell lines is preferred. For example, sequences encoding PROAP can be transformed into cell lines using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for about 1 to 2 days in enriched media before being switched to selective media. The purpose of the selectable marker is to
 20 confer resistance to a selective agent, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be propagated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase and adenine
 25 phosphoribosyltransferase genes, for use in *tk*⁻ and *aprt*⁻ cells, respectively. (See, e.g., Wigler, M. et al. (1977) Cell 11:223-232; Lowy, I. et al. (1980) Cell 22:817-823.) Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, *dhfr* confers resistance to methotrexate; *neo* confers resistance to the aminoglycosides neomycin and G-418; and *als* and *pat* confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively. (See, e.g.,
 30 Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. USA 77:3567-3570; Colbere-Garapin, F. et al. (1981) J. Mol. Biol. 150:1-14.) Additional selectable genes have been described, e.g., *trpB* and *hisD*, which alter cellular requirements for metabolites. (See, e.g., Hartman, S.C. and R.C. Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:8047-8051.) Visible markers, e.g., anthocyanins, green fluorescent proteins (GFP; Clontech), β glucuronidase and its substrate β -glucuronide, or luciferase and its substrate

luciferin may be used. These markers can be used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system. (See, e.g., Rhodes, C.A. (1995) *Methods Mol. Biol.* 55:121-131.)

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, the presence and expression of the gene may need to be confirmed. For example, if the sequence encoding PROAP is inserted within a marker gene sequence, transformed cells containing sequences encoding PROAP can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding PROAP under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

In general, host cells that contain the nucleic acid sequence encoding PROAP and that express PROAP may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations, PCR amplification, and protein bioassay or immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein sequences.

Immunological methods for detecting and measuring the expression of PROAP using either specific polyclonal or monoclonal antibodies are known in the art. Examples of such techniques include enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on PROAP is preferred, but a competitive binding assay may be employed. These and other assays are well known in the art. (See, e.g., Hampton, R. et al. (1990) Serological Methods, a Laboratory Manual, APS Press, St. Paul MN, Sect. IV; Coligan, J.E. et al. (1997) Current Protocols in Immunology, Greene Pub. Associates and Wiley-Interscience, New York NY; and Pound, J.D. (1998) Immunochemical Protocols, Humana Press, Totowa NJ.)

A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding PROAP include oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, the sequences encoding PROAP, or any fragments thereof, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits, such as those provided by Amersham Pharmacia Biotech, Promega

(Madison WI), and US Biochemical. Suitable reporter molecules or labels which may be used for ease of detection include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with nucleotide sequences encoding PROAP may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a transformed cell may be secreted or retained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode PROAP may be designed to contain signal sequences which direct secretion of PROAP through a prokaryotic or eukaryotic cell membrane.

In addition, a host cell strain may be chosen for its ability to modulate expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" or "pro" form of the protein may also be used to specify protein targeting, folding, and/or activity. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38) are available from the American Type Culture Collection (ATCC, Manassas VA) and may be chosen to ensure the correct modification and processing of the foreign protein.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences encoding PROAP may be ligated to a heterologous sequence resulting in translation of a fusion protein in any of the aforementioned host systems. For example, a chimeric PROAP protein containing a heterologous moiety that can be recognized by a commercially available antibody may facilitate the screening of peptide libraries for inhibitors of PROAP activity. Heterologous protein and peptide moieties may also facilitate purification of fusion proteins using commercially available affinity matrices. Such moieties include, but are not limited to, glutathione S-transferase (GST), maltose binding protein (MBP), thioredoxin (Trx), calmodulin binding peptide (CBP), 6-His, FLAG, *c-myc*, and hemagglutinin (HA). GST, MBP, Trx, CBP, and 6-His enable purification of their cognate fusion proteins on immobilized glutathione, maltose, phenylarsine oxide, calmodulin, and metal-chelate resins, respectively. FLAG, *c-myc*, and hemagglutinin (HA) enable immunoaffinity purification of fusion proteins using commercially available monoclonal and polyclonal antibodies that specifically recognize these epitope tags. A fusion protein may also be engineered to contain a proteolytic cleavage site located between the PROAP encoding sequence and the heterologous protein sequence, so that PROAP may be cleaved away from the heterologous moiety following purification. Methods for fusion protein expression and purification are discussed in Ausubel (1995, supra, ch. 10).

A variety of commercially available kits may also be used to facilitate expression and purification of fusion proteins.

In a further embodiment of the invention, synthesis of radiolabeled PROAP may be achieved in vitro using the TNT rabbit reticulocyte lysate or wheat germ extract system (Promega). These systems couple transcription and translation of protein-coding sequences operably associated with the T7, T3, or SP6 promoters. Translation takes place in the presence of a radiolabeled amino acid precursor, for example, ³⁵S-methionine.

Fragments of PROAP may be produced not only by recombinant means, but also by direct peptide synthesis using solid-phase techniques. (See, e.g., Creighton, supra, pp. 55-60.) Protein synthesis may be performed by manual techniques or by automation. Automated synthesis may be achieved, for example, using the ABI 431A peptide synthesizer (Perkin-Elmer). Various fragments of PROAP may be synthesized separately and then combined to produce the full length molecule.

THERAPEUTICS

Chemical and structural similarity, e.g., in the context of sequences and motifs, exists between regions of PROAP and proliferation and apoptosis related proteins. In addition, the expression of PROAP is closely associated with cancer, inflammation, and proliferating, reproductive, and developmental tissues. Therefore, PROAP appears to play a role in cell proliferative, immunological, and reproductive disorders. In the treatment of disorders associated with increased PROAP expression or activity, it is desirable to decrease the expression or activity of PROAP. In the treatment of disorders associated with decreased PROAP expression or activity, it is desirable to increase the expression or activity of PROAP.

Therefore, in one embodiment, PROAP or a fragment or derivative thereof may be administered to a subject to treat or prevent a disorder associated with decreased expression or activity of PROAP. Examples of such disorders include, but are not limited to, a cell proliferative disorder such as actinic keratosis, arteriosclerosis, atherosclerosis, bursitis, cirrhosis, hepatitis, mixed connective tissue disease (MCTD), myelofibrosis, paroxysmal nocturnal hemoglobinuria, polycythemia vera, psoriasis, primary thrombocythemia, and cancers including adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in particular, a cancer of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, penis, prostate, salivary glands, skin, spleen, testis, thymus, thyroid, and uterus; an immunological disorder such as acquired immunodeficiency syndrome (AIDS), Addison's disease, adult respiratory distress syndrome, allergies, ankylosing spondylitis, amyloidosis, anemia, asthma, atherosclerosis, autoimmune hemolytic anemia, autoimmune thyroiditis, autoimmune polyendocrinopathy-

candidiasis-ectodermal dystrophy (APECED), bronchitis, cholecystitis, contact dermatitis, Crohn's disease, atopic dermatitis, dermatomyositis, diabetes mellitus, emphysema, episodic lymphopenia with lymphocytotoxins, erythroblastosis fetalis, erythema nodosum, atrophic gastritis, glomerulonephritis, Goodpasture's syndrome, gout, Graves' disease, Hashimoto's thyroiditis, hypereosinophilia, irritable bowel syndrome, multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, osteoarthritis, osteoporosis, pancreatitis, polymyositis, psoriasis, Reiter's syndrome, rheumatoid arthritis, scleroderma, Sjögren's syndrome, systemic anaphylaxis, systemic lupus erythematosus, systemic sclerosis, thrombocytopenic purpura, ulcerative colitis, uveitis, Werner syndrome, a complication of cancer, hemodialysis, and extracorporeal circulation, viral, bacterial, fungal, parasitic, protozoal, and helminthic infections, and trauma; and a reproductive disorder such as disorders of prolactin production, infertility, including tubal disease, ovulatory defects, and endometriosis, disruptions of the estrous cycle, disruptions of the menstrual cycle, polycystic ovary syndrome, ovarian hyperstimulation syndrome, endometrial and ovarian tumors, uterine fibroids, autoimmune disorders, ectopic pregnancies, and teratogenesis; cancer of the breast, fibrocystic breast disease, and galactorrhea; disruptions of spermatogenesis, abnormal sperm physiology, cancer of the testis, cancer of the prostate, benign prostatic hyperplasia, prostatitis, Peyronie's disease, impotence, carcinoma of the male breast, and gynecomastia.

In another embodiment, a vector capable of expressing PROAP or a fragment or derivative thereof may be administered to a subject to treat or prevent a disorder associated with decreased expression or activity of PROAP including, but not limited to, those described above.

In a further embodiment, a pharmaceutical composition comprising a substantially purified PROAP in conjunction with a suitable pharmaceutical carrier may be administered to a subject to treat or prevent a disorder associated with decreased expression or activity of PROAP including, but not limited to, those provided above.

In still another embodiment, an agonist which modulates the activity of PROAP may be administered to a subject to treat or prevent a disorder associated with decreased expression or activity of PROAP including, but not limited to, those listed above.

In a further embodiment, an antagonist of PROAP may be administered to a subject to treat or prevent a disorder associated with increased expression or activity of PROAP. Examples of such disorders include, but are not limited to, those cell proliferative, immunological, and reproductive disorders described above. In one aspect, an antibody which specifically binds PROAP may be used directly as an antagonist or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissues which express PROAP.

In an additional embodiment, a vector expressing the complement of the polynucleotide

encoding PROAP may be administered to a subject to treat or prevent a disorder associated with increased expression or activity of PROAP including, but not limited to, those described above.

In other embodiments, any of the proteins, antagonists, antibodies, agonists, complementary sequences, or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

An antagonist of PROAP may be produced using methods which are generally known in the art. In particular, purified PROAP may be used to produce antibodies or to screen libraries of pharmaceutical agents to identify those which specifically bind PROAP. Antibodies to PROAP may also be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, and single chain antibodies, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies (i.e., those which inhibit dimer formation) are generally preferred for therapeutic use.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others may be immunized by injection with PROAP or with any fragment or oligopeptide thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, KLH, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially preferable.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to PROAP have an amino acid sequence consisting of at least about 5 amino acids, and generally will consist of at least about 10 amino acids. It is also preferable that these oligopeptides, peptides, or fragments are identical to a portion of the amino acid sequence of the natural protein and contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of PROAP amino acids may be fused with those of another protein, such as KLH, and antibodies to the chimeric molecule may be produced.

Monoclonal antibodies to PROAP may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique. (See, e.g., Kohler, G. et al. (1975) Nature 256:495-497; Kozbor, D. et al. (1985) J.

Immunol. Methods 81:31-42; Cote, R.J. et al. (1983) Proc. Natl. Acad. Sci. USA 80:2026-2030; and Cole, S.P. et al. (1984) Mol. Cell Biol. 62:109-120.)

In addition, techniques developed for the production of "chimeric antibodies," such as the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used. (See, e.g., Morrison, S.L. et al. (1984) Proc. Natl. Acad. Sci. USA 81:6851-6855; Neuberger, M.S. et al. (1984) Nature 312:604-608; and Takeda, S. et al. (1985) Nature 314:452-454.) Alternatively, techniques described for the production of single chain antibodies may be adapted, using methods known in the art, to produce PROAP-specific single chain antibodies. Antibodies with related specificity, but of distinct idiotypic composition, may be generated by chain shuffling from random combinatorial immunoglobulin libraries. (See, e.g., Burton, D.R. (1991) Proc. Natl. Acad. Sci. USA 88:10134-10137.)

Antibodies may also be produced by inducing in vivo production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature. (See, e.g., Orlandi, R. et al. (1989) Proc. Natl. Acad. Sci. USA 86:3833-3837; Winter, G. et al. (1991) Nature 349:293-299.)

Antibody fragments which contain specific binding sites for PROAP may also be generated. For example, such fragments include, but are not limited to, F(ab')₂ fragments produced by pepsin digestion of the antibody molecule and Fab fragments generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (See, e.g., Huse, W.D. et al. (1989) Science 246:1275-1281.)

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between PROAP and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering PROAP epitopes is generally used, but a competitive binding assay may also be employed (Pound, supra).

Various methods such as Scatchard analysis in conjunction with radioimmunoassay techniques may be used to assess the affinity of antibodies for PROAP. Affinity is expressed as an association constant, K_a , which is defined as the molar concentration of PROAP-antibody complex divided by the molar concentrations of free antigen and free antibody under equilibrium conditions. The K_a determined for a preparation of polyclonal antibodies, which are heterogeneous in their affinities for multiple PROAP epitopes, represents the average affinity, or avidity, of the antibodies

for PROAP. The K_a determined for a preparation of monoclonal antibodies, which are monospecific for a particular PROAP epitope, represents a true measure of affinity. High-affinity antibody preparations with K_a ranging from about 10^9 to 10^{12} L/mole are preferred for use in immunoassays in which the PROAP-antibody complex must withstand rigorous manipulations. Low-affinity antibody
5 preparations with K_a ranging from about 10^6 to 10^7 L/mole are preferred for use in immunopurification and similar procedures which ultimately require dissociation of PROAP, preferably in active form, from the antibody (Catty, D. (1988) Antibodies, Volume I: A Practical Approach, IRL Press, Washington, DC; Liddell, J.E. and Cryer, A. (1991) A Practical Guide to Monoclonal Antibodies, John Wiley & Sons, New York NY).

10 The titer and avidity of polyclonal antibody preparations may be further evaluated to determine the quality and suitability of such preparations for certain downstream applications. For example, a polyclonal antibody preparation containing at least 1-2 mg specific antibody/ml, preferably 5-10 mg specific antibody/ml, is generally employed in procedures requiring precipitation of PROAP-antibody complexes. Procedures for evaluating antibody specificity, titer, and avidity, and
15 guidelines for antibody quality and usage in various applications, are generally available. (See, e.g., Catty, supra, and Coligan et al. supra.)

In another embodiment of the invention, the polynucleotides encoding PROAP, or any fragment or complement thereof, may be used for therapeutic purposes. In one aspect, the complement of the polynucleotide encoding PROAP may be used in situations in which it would be
20 desirable to block the transcription of the mRNA. In particular, cells may be transformed with sequences complementary to polynucleotides encoding PROAP. Thus, complementary molecules or fragments may be used to modulate PROAP activity, or to achieve regulation of gene function. Such technology is now well known in the art, and sense or antisense oligonucleotides or larger fragments can be designed from various locations along the coding or control regions of sequences encoding
25 PROAP.

Expression vectors derived from retroviruses, adenoviruses, or herpes or vaccinia viruses, or from various bacterial plasmids, may be used for delivery of nucleotide sequences to the targeted organ, tissue, or cell population. Methods which are well known to those skilled in the art can be used to construct vectors to express nucleic acid sequences complementary to the polynucleotides
30 encoding PROAP. (See, e.g., Sambrook, supra; Ausubel, 1995, supra.)

Genes encoding PROAP can be turned off by transforming a cell or tissue with expression vectors which express high levels of a polynucleotide, or fragment thereof, encoding PROAP. Such constructs may be used to introduce untranslatable sense or antisense sequences into a cell. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules

until they are disabled by endogenous nucleases. Transient expression may last for a month or more with a non-replicating vector, and may last even longer if appropriate replication elements are part of the vector system.

As mentioned above, modifications of gene expression can be obtained by designing complementary sequences or antisense molecules (DNA, RNA, or PNA) to the control, 5', or regulatory regions of the gene encoding PROAP. Oligonucleotides derived from the transcription initiation site, e.g., between about positions -10 and +10 from the start site, may be employed. Similarly, inhibition can be achieved using triple helix base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA have been described in the literature. (See, e.g., Gee, J.E. et al. (1994) in Huber, B.E. and B.I. Carr, Molecular and Immunologic Approaches, Futura Publishing, Mt. Kisco NY, pp. 163-177.) A complementary sequence or antisense molecule may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to catalyze the specific cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. For example, engineered hammerhead motif ribozyme molecules may specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding PROAP.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, including the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides, corresponding to the region of the target gene containing the cleavage site, may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Complementary ribonucleic acid molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis.

Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding PROAP. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, these cDNA constructs that synthesize complementary RNA, constitutively or inducibly, can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept is inherent in the production of PNAs and can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

Many methods for introducing vectors into cells or tissues are available and equally suitable for use in vivo, in vitro, and ex vivo. For ex vivo therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection, by liposome injections, or by polycationic amino polymers may be achieved using methods which are well known in the art. (See, e.g., Goldman, C.K. et al. (1997) Nat. Biotechnol. 15:462-466.)

Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as humans, dogs, cats, cows, horses, rabbits, and monkeys.

An additional embodiment of the invention relates to the administration of a pharmaceutical or sterile composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions may consist of PROAP, antibodies to PROAP, and mimetics, agonists, antagonists, or inhibitors of PROAP. The compositions may be administered alone or in combination with at least one other agent, such as a stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs, or hormones.

The pharmaceutical compositions utilized in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing, Easton PA).

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

5 Pharmaceutical preparations for oral use can be obtained through combining active compounds with solid excipient and processing the resultant mixture of granules (optionally, after grinding) to obtain tablets or dragee cores. Suitable auxiliaries can be added, if desired. Suitable excipients include carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, and sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, 10 hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums, including arabic and tragacanth; and proteins, such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, and alginic acid or a salt thereof, such as sodium alginate.

Dragee cores may be used in conjunction with suitable coatings, such as concentrated sugar 15 solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of 20 gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with fillers or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

25 Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily 30 injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate, triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents to increase the solubility of the compounds and allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating,

5 dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, and succinic acids. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preparation may be a lyophilized powder which may contain any
10 or all of the following: 1 mM to 50 mM histidine, 0.1% to 2% sucrose, and 2% to 7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For administration of PROAP, such labeling would include amount, frequency, and method of administration.

15 Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models such as mice, rats, rabbits, dogs, or pigs.

20 An animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, for example PROAP or fragments thereof, antibodies of PROAP, and agonists, antagonists or inhibitors of
25 PROAP, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or with experimental animals, such as by calculating the ED_{50} (the dose therapeutically effective in 50% of the population) or LD_{50} (the dose lethal to 50% of the population) statistics. The dose ratio of toxic to therapeutic effects is the therapeutic index, which can be expressed as the LD_{50}/ED_{50} ratio. Pharmaceutical compositions
30 which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies are used to formulate a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that includes the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, the sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject requiring treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, the general health of the subject, the age, weight, and gender of the subject, time and frequency of administration, drug combination(s), reaction sensitivities, and response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or biweekly depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from about 0.1 μg to 100,000 μg , up to a total dose of about 1 gram, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

DIAGNOSTICS

In another embodiment, antibodies which specifically bind PROAP may be used for the diagnosis of disorders characterized by expression of PROAP, or in assays to monitor patients being treated with PROAP or agonists, antagonists, or inhibitors of PROAP. Antibodies useful for diagnostic purposes may be prepared in the same manner as described above for therapeutics.

Diagnostic assays for PROAP include methods which utilize the antibody and a label to detect PROAP in human body fluids or in extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by covalent or non-covalent attachment of a reporter molecule. A wide variety of reporter molecules, several of which are described above, are known in the art and may be used.

A variety of protocols for measuring PROAP, including ELISAs, RIAs, and FACS, are known in the art and provide a basis for diagnosing altered or abnormal levels of PROAP expression. Normal or standard values for PROAP expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, for example, human subjects, with antibody to PROAP under conditions suitable for complex formation. The amount of standard complex formation may be quantitated by various methods, such as photometric means. Quantities of PROAP expressed in subject, control, and disease samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding PROAP may be used

for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, complementary RNA and DNA molecules, and PNAs. The polynucleotides may be used to detect and quantify gene expression in biopsied tissues in which expression of PROAP may be correlated with disease. The diagnostic assay may be used to determine absence, presence, and excess
 5 expression of PROAP, and to monitor regulation of PROAP levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding PROAP or closely related molecules may be used to identify nucleic acid sequences which encode PROAP. The specificity of the probe, whether it is made from a highly specific region, e.g., the 5' regulatory region, or from a less specific region, e.g., a
 10 conserved motif, and the stringency of the hybridization or amplification will determine whether the probe identifies only naturally occurring sequences encoding PROAP, allelic variants, or related sequences.

Probes may also be used for the detection of related sequences, and may have at least 50% sequence identity to any of the PROAP encoding sequences. The hybridization probes of the subject
 15 invention may be DNA or RNA and may be derived from the sequence of SEQ ID NO:20-38 or from genomic sequences including promoters, enhancers, and introns of the PROAP gene.

Means for producing specific hybridization probes for DNAs encoding PROAP include the cloning of polynucleotide sequences encoding PROAP or PROAP derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, are commercially available, and may
 20 be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example, by radionuclides such as ^{32}P or ^{35}S , or by enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

Polynucleotide sequences encoding PROAP may be used for the diagnosis of disorders
 25 associated with expression of PROAP. Examples of such disorders include, but are not limited to, a cell proliferative disorder such as actinic keratosis, arteriosclerosis, atherosclerosis, bursitis, cirrhosis, hepatitis, mixed connective tissue disease (MCTD), myelofibrosis, paroxysmal nocturnal hemoglobinuria, polycythemia vera, psoriasis, primary thrombocythemia, and cancers including adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in
 30 particular, a cancer of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, penis, prostate, salivary glands, skin, spleen, testis, thymus, thyroid, and uterus; an immunological disorder such as acquired immunodeficiency syndrome (AIDS), Addison's disease, adult respiratory distress syndrome, allergies, ankylosing spondylitis, amyloidosis, anemia, asthma, atherosclerosis,

autoimmune hemolytic anemia, autoimmune thyroiditis, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), bronchitis, cholecystitis, contact dermatitis, Crohn's disease, atopic dermatitis, dermatomyositis, diabetes mellitus, emphysema, episodic lymphopenia with lymphocytotoxins, erythroblastosis fetalis, erythema nodosum, atrophic gastritis,

5 glomerulonephritis, Goodpasture's syndrome, gout, Graves' disease, Hashimoto's thyroiditis, hypereosinophilia, irritable bowel syndrome, multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, osteoarthritis, osteoporosis, pancreatitis, polymyositis, psoriasis, Reiter's syndrome, rheumatoid arthritis, scleroderma, Sjögren's syndrome, systemic anaphylaxis, systemic lupus erythematosus, systemic sclerosis, thrombocytopenic purpura, ulcerative colitis, uveitis, Werner

10 syndrome, a complication of cancer, hemodialysis, and extracorporeal circulation, viral, bacterial, fungal, parasitic, protozoal, and helminthic infections, and trauma; and a reproductive disorder such as disorders of prolactin production, infertility, including tubal disease, ovulatory defects, and endometriosis, disruptions of the estrous cycle, disruptions of the menstrual cycle, polycystic ovary syndrome, ovarian hyperstimulation syndrome, endometrial and ovarian tumors, uterine fibroids,

15 autoimmune disorders, ectopic pregnancies, and teratogenesis; cancer of the breast, fibrocystic breast disease, and galactorrhea; disruptions of spermatogenesis, abnormal sperm physiology, cancer of the testis, cancer of the prostate, benign prostatic hyperplasia, prostatitis, Peyronie's disease, impotence, carcinoma of the male breast, and gynecomastia. The polynucleotide sequences encoding PROAP may be used in Southern or northern analysis, dot blot, or other membrane-based technologies; in

20 PCR technologies; in dipstick, pin, and multiformat ELISA-like assays; and in microarrays utilizing fluids or tissues from patients to detect altered PROAP expression. Such qualitative or quantitative methods are well known in the art.

In a particular aspect, the nucleotide sequences encoding PROAP may be useful in assays that detect the presence of associated disorders, particularly those mentioned above. The nucleotide

25 sequences encoding PROAP may be labeled by standard methods and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantified and compared with a standard value. If the amount of signal in the patient sample is significantly altered in comparison to a control sample then the presence of altered levels of nucleotide sequences encoding PROAP in the

30 sample indicates the presence of the associated disorder. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or to monitor the treatment of an individual patient.

In order to provide a basis for the diagnosis of a disorder associated with expression of PROAP, a normal or standard profile for expression is established. This may be accomplished by

combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, encoding PROAP, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with values from an experiment in which a known amount of a substantially purified polynucleotide is used. Standard values obtained in this manner may be compared with values obtained from samples from patients who are symptomatic for a disorder. Deviation from standard values is used to establish the presence of a disorder.

Once the presence of a disorder is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to determine if the level of expression in the patient begins to approximate that which is observed in the normal subject. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

With respect to cancer, the presence of an abnormal amount of transcript (either under- or overexpressed) in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Additional diagnostic uses for oligonucleotides designed from the sequences encoding PROAP may involve the use of PCR. These oligomers may be chemically synthesized, generated enzymatically, or produced *in vitro*. Oligomers will preferably contain a fragment of a polynucleotide encoding PROAP, or a fragment of a polynucleotide complementary to the polynucleotide encoding PROAP, and will be employed under optimized conditions for identification of a specific gene or condition. Oligomers may also be employed under less stringent conditions for detection or quantification of closely related DNA or RNA sequences.

Methods which may also be used to quantify the expression of PROAP include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and interpolating results from standard curves. (See, e.g., Melby, P.C. et al. (1993) J. Immunol. Methods 159:235-244; Duplaa, C. et al. (1993) Anal. Biochem. 212:229-236.) The speed of quantitation of multiple samples may be accelerated by running the assay in a high-throughput format where the oligomer of interest is presented in various dilutions and a spectrophotometric or colorimetric response gives rapid quantitation.

In further embodiments, oligonucleotides or longer fragments derived from any of the polynucleotide sequences described herein may be used as targets in a microarray. The microarray

can be used to monitor the expression level of large numbers of genes simultaneously and to identify genetic variants, mutations, and polymorphisms. This information may be used to determine gene function, to understand the genetic basis of a disorder, to diagnose a disorder, and to develop and monitor the activities of therapeutic agents.

5 Microarrays may be prepared, used, and analyzed using methods known in the art. (See, e.g., Brennan, T.M. et al. (1995) U.S. Patent No. 5,474,796; Schena, M. et al. (1996) Proc. Natl. Acad. Sci. USA 93:10614-10619; Baldeschweiler et al. (1995) PCT application WO95/251116; Shalon, D. et al. (1995) PCT application WO95/35505; Heller, R.A. et al. (1997) Proc. Natl. Acad. Sci. USA 94:2150-2155; and Heller, M.J. et al. (1997) U.S. Patent No. 5,605,662.)

10 In another embodiment of the invention, nucleic acid sequences encoding PROAP may be used to generate hybridization probes useful in mapping the naturally occurring genomic sequence. The sequences may be mapped to a particular chromosome, to a specific region of a chromosome, or to artificial chromosome constructions, e.g., human artificial chromosomes (HACs), yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), bacterial Pl constructions, or single
15 chromosome cDNA libraries. (See, e.g., Harrington, J.J. et al. (1997) Nat. Genet. 15:345-355; Price, C.M. (1993) Blood Rev. 7:127-134; and Trask, B.J. (1991) Trends Genet. 7:149-154.)

Fluorescent in situ hybridization (FISH) may be correlated with other physical chromosome mapping techniques and genetic map data. (See, e.g., Heinz-Ulrich, et al. (1995) in Meyers, supra, pp. 965-968.) Examples of genetic map data can be found in various scientific journals or at the
20 Online Mendelian Inheritance in Man (OMIM) World Wide Web site. Correlation between the location of the gene encoding PROAP on a physical chromosomal map and a specific disorder, or a predisposition to a specific disorder, may help define the region of DNA associated with that disorder. The nucleotide sequences of the invention may be used to detect differences in gene sequences among normal, carrier, and affected individuals.

25 In situ hybridization of chromosomal preparations and physical mapping techniques, such as linkage analysis using established chromosomal markers, may be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms by physical mapping. This provides
30 valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region, e.g., ataxia-telangiectasia to 11q22-23, any sequences mapping to that area may represent associated or regulatory genes for further investigation. (See, e.g., Gatti, R.A. et al. (1988) Nature 336:577-580.) The nucleotide sequence of the subject invention

may also be used to detect differences in the chromosomal location due to translocation, inversion, etc., among normal, carrier, or affected individuals.

In another embodiment of the invention, PROAP, its catalytic or immunogenic fragments, or oligopeptides thereof can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes between PROAP and the agent being tested may be measured.

Another technique for drug screening provides for high throughput screening of compounds having suitable binding affinity to the protein of interest. (See, e.g., Geysen, et al. (1984) PCT application WO84/03564.) In this method, large numbers of different small test compounds are synthesized on a solid substrate. The test compounds are reacted with PROAP, or fragments thereof, and washed. Bound PROAP is then detected by methods well known in the art. Purified PROAP can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding PROAP specifically compete with a test compound for binding PROAP. In this manner, antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with PROAP.

In additional embodiments, the nucleotide sequences which encode PROAP may be used in any molecular biology techniques that have yet to be developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including, but not limited to, such properties as the triplet genetic code and specific base pair interactions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The disclosures of all patents, applications, and publications mentioned above and below, in particular U.S. Ser. No. [Atty Docket No. PF-0619 P, filed October 20, 1998] U.S. Ser. No. 60/118,559, U.S. Ser. No. [Atty Docket No. PF-0670 P, filed February 11, 1999], and U.S. Ser. No. 60/154,336 are hereby expressly incorporated by reference.

EXAMPLES

I. Construction of cDNA Libraries

RNA was purchased from Clontech or isolated from tissues described in Table 4. Some tissues were homogenized and lysed in guanidinium isothiocyanate, while others were homogenized and lysed in phenol or in a suitable mixture of denaturants, such as TRIZOL (Life Technologies), a monophasic solution of phenol and guanidine isothiocyanate. The resulting lysates were centrifuged over CsCl cushions or extracted with chloroform. RNA was precipitated from the lysates with either isopropanol or sodium acetate and ethanol, or by other routine methods.

Phenol extraction and precipitation of RNA were repeated as necessary to increase RNA purity. In some cases, RNA was treated with DNase. For most libraries, poly(A⁺) RNA was isolated using oligo d(T)-coupled paramagnetic particles (Promega), OLIGOTEX latex particles (QIAGEN, Chatsworth CA), or an OLIGOTEX mRNA purification kit (QIAGEN). Alternatively, RNA was isolated directly from tissue lysates using other RNA isolation kits, e.g., the POLY(A)PURE mRNA purification kit (Ambion, Austin TX).

In some cases, Stratagene was provided with RNA and constructed the corresponding cDNA libraries. Otherwise, cDNA was synthesized and cDNA libraries were constructed with the UNIZAP vector system (Stratagene) or SUPERScript plasmid system (Life Technologies), using the recommended procedures or similar methods known in the art. (See, e.g., Ausubel, 1997, *supra*, units 5.1-6.6.) Reverse transcription was initiated using oligo d(T) or random primers. Synthetic oligonucleotide adapters were ligated to double stranded cDNA, and the cDNA was digested with the appropriate restriction enzyme or enzymes. For most libraries, the cDNA was size-selected (300-1000 bp) using SEPHACRYL S1000, SEPHAROSE CL2B, or SEPHAROSE CL4B column chromatography (Amersham Pharmacia Biotech) or preparative agarose gel electrophoresis. cDNAs were ligated into compatible restriction enzyme sites of the polylinker of a suitable plasmid, e.g., PBLUESCRIPT plasmid (Stratagene), PSORT1 plasmid (Life Technologies), or pINCY (Incyte Pharmaceuticals, Palo Alto CA). Recombinant plasmids were transformed into competent *E. coli* cells including XLI-Blue, XLI-BlueMRF, or SOLR from Stratagene or DH5 α , DH10B, or ElectroMAX DH10B from Life Technologies.

II. Isolation of cDNA Clones

Plasmids were recovered from host cells by *in vivo* excision using the UNIZAP vector system (Stratagene) or by cell lysis. Plasmids were purified using at least one of the following: a Magic or WIZARD Minipreps DNA purification system (Promega); an AGTC Miniprep purification kit (Edge Biosystems, Gaithersburg MD); and QIAWELL 8 Plasmid, QIAWELL 8 Plus Plasmid, QIAWELL 8 Ultra Plasmid purification systems or the R.E.A.L. PREP 96 plasmid purification kit

from QIAGEN. Following precipitation, plasmids were resuspended in 0.1 ml of distilled water and stored, with or without lyophilization, at 4°C.

Alternatively, plasmid DNA was amplified from host cell lysates using direct link PCR in a high-throughput format (Rao, V.B. (1994) Anal. Biochem. 216:1-14). Host cell lysis and thermal cycling steps were carried out in a single reaction mixture. Samples were processed and stored in 384-well plates, and the concentration of amplified plasmid DNA was quantified fluorometrically using PICOGREEN dye (Molecular Probes, Eugene OR) and a FLUOROSKAN II fluorescence scanner (Labsystems Oy, Helsinki, Finland).

III. Sequencing and Analysis

cDNA sequencing reactions were processed using standard methods or high-throughput instrumentation such as the ABI CATALYST 800 (Perkin-Elmer) thermal cycler or the PTC-200 thermal cycler (MJ Research) in conjunction with the HYDRA microdispenser (Robbins Scientific) or the MICROLAB 2200 (Hamilton) liquid transfer system. cDNA sequencing reactions were prepared using reagents provided by Amersham Pharmacia Biotech or supplied in ABI sequencing kits such as the ABI PRISM BIGDYE Terminator cycle sequencing ready reaction kit (Perkin-Elmer). Electrophoretic separation of cDNA sequencing reactions and detection of labeled polynucleotides were carried out using the MEGABACE 1000 DNA sequencing system (Molecular Dynamics); the ABI PRISM 373 or 377 sequencing system (Perkin-Elmer) in conjunction with standard ABI protocols and base calling software; or other sequence analysis systems known in the art. Reading frames within the cDNA sequences were identified using standard methods (reviewed in Ausubel, 1997, supra, unit 7.7). Some of the cDNA sequences were selected for extension using the techniques disclosed in Example V.

The polynucleotide sequences derived from cDNA sequencing were assembled and analyzed using a combination of software programs which utilize algorithms well known to those skilled in the art. Table 5 summarizes the tools, programs, and algorithms used and provides applicable descriptions, references, and threshold parameters. The first column of Table 5 shows the tools, programs, and algorithms used, the second column provides brief descriptions thereof, the third column presents appropriate references, all of which are incorporated by reference herein in their entirety, and the fourth column presents, where applicable, the scores, probability values, and other parameters used to evaluate the strength of a match between two sequences (the higher the score, the greater the homology between two sequences). Sequences were analyzed using MACDNASIS PRO software (Hitachi Software Engineering, South San Francisco CA) and LASERGENE software (DNASTAR). Polynucleotide and polypeptide sequence alignments were generated using the default parameters specified by the clustal algorithm as incorporated into the MEGALIGN multisequence

alignment program (DNASTAR), which also calculates the percent identity between aligned sequences.

The polynucleotide sequences were validated by removing vector, linker, and polyA sequences and by masking ambiguous bases, using algorithms and programs based on BLAST, dynamic programming, and dinucleotide nearest neighbor analysis. The sequences were then queried against a selection of public databases such as the GenBank primate, rodent, mammalian, vertebrate, and eukaryote databases, and BLOCKS, PRINTS, DOMO, PRODOM, and PFAM to acquire annotation using programs based on BLAST, FASTA, and BLIMPS. The sequences were assembled into full length polynucleotide sequences using programs based on Phred, Phrap, and Consed, and were screened for open reading frames using programs based on GeneMark, BLAST, and FASTA. The full length polynucleotide sequences were translated to derive the corresponding full length amino acid sequences, and these full length sequences were subsequently analyzed by querying against databases such as the GenBank databases (described above), SwissProt, BLOCKS, PRINTS, DOMO, PRODOM, Prosite, and Hidden Markov Model (HMM)-based protein family databases such as PFAM. HMM is a probabilistic approach which analyzes consensus primary structures of gene families. (See, e.g., Eddy, S.R. (1996) Curr. Opin. Struct. Biol. 6:361-365.)

The programs described above for the assembly and analysis of full length polynucleotide and amino acid sequences were also used to identify polynucleotide sequence fragments from SEQ ID NO:20-38. Fragments from about 20 to about 4000 nucleotides which are useful in hybridization and amplification technologies were described in The Invention section above.

IV. Northern Analysis

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which RNAs from a particular cell type or tissue have been bound. (See, e.g., Sambrook, supra, ch. 7; Ausubel, 1995, supra, ch. 4 and 16.)

Analogous computer techniques applying BLAST were used to search for identical or related molecules in nucleotide databases such as GenBank or LIFESEQ (Incyte Pharmaceuticals). This analysis is much faster than multiple membrane-based hybridizations. In addition, the sensitivity of the computer search can be modified to determine whether any particular match is categorized as exact or similar. The basis of the search is the product score, which is defined as:

$$\frac{\% \text{ sequence identity} \times \% \text{ maximum BLAST score}}{100}$$

100

The product score takes into account both the degree of similarity between two sequences and the length of the sequence match. For example, with a product score of 40, the match will be exact

within a 1% to 2% error, and, with a product score of 70, the match will be exact. Similar molecules are usually identified by selecting those which show product scores between 15 and 40, although lower scores may identify related molecules.

The results of northern analyses are reported as a percentage distribution of libraries in which the transcript encoding PROAP occurred. Analysis involved the categorization of cDNA libraries by organ/tissue and disease. The organ/tissue categories included cardiovascular, dermatologic, developmental, endocrine, gastrointestinal, hematopoietic/immune, musculoskeletal, nervous, reproductive, and urologic. The disease/condition categories included cancer, inflammation/trauma, cell proliferation, neurological, and pooled. For each category, the number of libraries expressing the sequence of interest was counted and divided by the total number of libraries across all categories. Percentage values of tissue-specific and disease- or condition-specific expression are reported in Table 3.

V. Extension of PROAP Encoding Polynucleotides

The full length nucleic acid sequences of SEQ ID NO:20-38 were produced by extension of an appropriate fragment of the full length molecule using oligonucleotide primers designed from this fragment. One primer was synthesized to initiate 5' extension of the known fragment, and the other primer, to initiate 3' extension of the known fragment. The initial primers were designed using OLIGO 4.06 software (National Biosciences), or another appropriate program, to be about 22 to 30 nucleotides in length, to have a GC content of about 50% or more, and to anneal to the target sequence at temperatures of about 68°C to about 72°C. Any stretch of nucleotides which would result in hairpin structures and primer-primer dimerizations was avoided.

Selected human cDNA libraries were used to extend the sequence. If more than one extension was necessary or desired, additional or nested sets of primers were designed.

High fidelity amplification was obtained by PCR using methods well known in the art. PCR was performed in 96-well plates using the PTC-200 thermal cycler (MJ Research, Inc.). The reaction mix contained DNA template, 200 nmol of each primer, reaction buffer containing Mg^{2+} , $(NH_4)_2SO_4$, and β -mercaptoethanol, Taq DNA polymerase (Amersham Pharmacia Biotech), ELONGASE enzyme (Life Technologies), and Pfu DNA polymerase (Stratagene), with the following parameters for primer pair PCI A and PCI B: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 60°C, 1 min; Step 4: 68°C, 2 min; Step 5: Steps 2, 3, and 4 repeated 20 times; Step 6: 68°C, 5 min; Step 7: storage at 4°C. In the alternative, the parameters for primer pair T7 and SK+ were as follows: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 57°C, 1 min; Step 4: 68°C, 2 min; Step 5: Steps 2, 3, and 4 repeated 20 times; Step 6: 68°C, 5 min; Step 7: storage at 4°C.

The concentration of DNA in each well was determined by dispensing 100 μ l PICOGREEN

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quantitation reagent (0.25% (v/v) PICOGREEN; Molecular Probes, Eugene OR) dissolved in 1X TE and 0.5 μ l of undiluted PCR product into each well of an opaque fluorimeter plate (Corning Costar, Acton MA), allowing the DNA to bind to the reagent. The plate was scanned in a Fluoroskan II (Labsystems Oy, Helsinki, Finland) to measure the fluorescence of the sample and to quantify the concentration of DNA. A 5 μ l to 10 μ l aliquot of the reaction mixture was analyzed by electrophoresis on a 1 % agarose mini-gel to determine which reactions were successful in extending the sequence.

The extended nucleotides were desalted and concentrated, transferred to 384-well plates, digested with CviJI cholera virus endonuclease (Molecular Biology Research, Madison WI), and sonicated or sheared prior to religation into pUC 18 vector (Amersham Pharmacia Biotech). For shotgun sequencing, the digested nucleotides were separated on low concentration (0.6 to 0.8%) agarose gels, fragments were excised, and agar digested with Agar ACE (Promega). Extended clones were religated using T4 ligase (New England Biolabs, Beverly MA) into pUC 18 vector (Amersham Pharmacia Biotech), treated with Pfu DNA polymerase (Stratagene) to fill-in restriction site overhangs, and transfected into competent *E. coli* cells. Transformed cells were selected on antibiotic-containing media, individual colonies were picked and cultured overnight at 37°C in 384-well plates in LB/2x carb liquid media.

The cells were lysed, and DNA was amplified by PCR using Taq DNA polymerase (Amersham Pharmacia Biotech) and Pfu DNA polymerase (Stratagene) with the following parameters: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 60°C, 1 min; Step 4: 72°C, 2 min; Step 5: steps 2, 3, and 4 repeated 29 times; Step 6: 72°C, 5 min; Step 7: storage at 4°C. DNA was quantified by PICOGREEN reagent (Molecular Probes) as described above. Samples with low DNA recoveries were reamplified using the same conditions as described above. Samples were diluted with 20% dimethylsulfoxide (1:2, v/v), and sequenced using DYENAMIC energy transfer sequencing primers and the DYENAMIC DIRECT kit (Amersham Pharmacia Biotech) or the ABI PRISM BIGDYE Terminator cycle sequencing ready reaction kit (Perkin-Elmer).

In like manner, the nucleotide sequences of SEQ ID NO:20-38 are used to obtain 5' regulatory sequences using the procedure above, oligonucleotides designed for such extension, and an appropriate genomic library.

VI. Labeling and Use of Individual Hybridization Probes

Hybridization probes derived from SEQ ID NO:20-38 are employed to screen cDNAs, genomic DNAs, or mRNAs. Although the labeling of oligonucleotides, consisting of about 20 base pairs, is specifically described, essentially the same procedure is used with larger nucleotide fragments. Oligonucleotides are designed using state-of-the-art software such as OLIGO 4.06

software (National Biosciences) and labeled by combining 50 pmol of each oligomer, 250 μ Ci of [γ - 32 P] adenosine triphosphate (Amersham Pharmacia Biotech), and T4 polynucleotide kinase (DuPont NEN, Boston MA). The labeled oligonucleotides are substantially purified using a SEPHADEX G-25 superfine size exclusion dextran bead column (Amersham Pharmacia Biotech).

- 5 An aliquot containing 10^7 counts per minute of the labeled probe is used in a typical membrane-based hybridization analysis of human genomic DNA digested with one of the following endonucleases: Ase I, Bgl II, Eco RI, Pst I, Xba I, or Pvu II (DuPont NEN).

The DNA from each digest is fractionated on a 0.7% agarose gel and transferred to nylon membranes (Nytran Plus, Schleicher & Schuell, Durham NH). Hybridization is carried out for 16
10 hours at 40°C. To remove nonspecific signals, blots are sequentially washed at room temperature under conditions of up to, for example, 0.1 x saline sodium citrate and 0.5% sodium dodecyl sulfate. Hybridization patterns are visualized using autoradiography or an alternative imaging means and compared.

VII. Microarrays

- 15 A chemical coupling procedure and an ink jet device can be used to synthesize array elements on the surface of a substrate. (See, e.g., Baldeschweiler, supra.) An array analogous to a dot or slot blot may also be used to arrange and link elements to the surface of a substrate using thermal, UV, chemical, or mechanical bonding procedures. A typical array may be produced by hand or using available methods and machines and contain any appropriate number of elements. After
20 hybridization, nonhybridized probes are removed and a scanner used to determine the levels and patterns of fluorescence. The degree of complementarity and the relative abundance of each probe which hybridizes to an element on the microarray may be assessed through analysis of the scanned images.

- Full-length cDNAs, Expressed Sequence Tags (ESTs), or fragments thereof may comprise
25 the elements of the microarray. Fragments suitable for hybridization can be selected using software well known in the art such as LASERGENE software (DNASTAR). Full-length cDNAs, ESTs, or fragments thereof corresponding to one of the nucleotide sequences of the present invention, or selected at random from a cDNA library relevant to the present invention, are arranged on an appropriate substrate, e.g., a glass slide. The cDNA is fixed to the slide using, e.g., UV cross-linking
30 followed by thermal and chemical treatments and subsequent drying. (See, e.g., Schena, M. et al. (1995) Science 270:467-470; Shalon, D. et al. (1996) Genome Res. 6:639-645.) Fluorescent probes are prepared and used for hybridization to the elements on the substrate. The substrate is analyzed by procedures described above.

VIII. Complementary Polynucleotides

Sequences complementary to the PROAP-encoding sequences, or any parts thereof, are used to detect, decrease, or inhibit expression of naturally occurring PROAP. Although use of oligonucleotides comprising from about 15 to 30 base pairs is described, essentially the same procedure is used with smaller or with larger sequence fragments. Appropriate oligonucleotides are designed using OLIGO 4.06 software (National Biosciences) and the coding sequence of PROAP. To inhibit transcription, a complementary oligonucleotide is designed from the most unique 5' sequence and used to prevent promoter binding to the coding sequence. To inhibit translation, a complementary oligonucleotide is designed to prevent ribosomal binding to the PROAP-encoding transcript.

IX. Expression of PROAP

Expression and purification of PROAP is achieved using bacterial or virus-based expression systems. For expression of PROAP in bacteria, cDNA is subcloned into an appropriate vector containing an antibiotic resistance gene and an inducible promoter that directs high levels of cDNA transcription. Examples of such promoters include, but are not limited to, the *trp-lac (tac)* hybrid promoter and the T5 or T7 bacteriophage promoter in conjunction with the *lac* operator regulatory element. Recombinant vectors are transformed into suitable bacterial hosts, e.g., BL21(DE3).

Antibiotic resistant bacteria express PROAP upon induction with isopropyl beta-D-thiogalactopyranoside (IPTG). Expression of PROAP in eukaryotic cells is achieved by infecting insect or mammalian cell lines with recombinant Autographica californica nuclear polyhedrosis virus (AcMNPV), commonly known as baculovirus. The nonessential polyhedrin gene of baculovirus is replaced with cDNA encoding PROAP by either homologous recombination or bacterial-mediated transposition involving transfer plasmid intermediates. Viral infectivity is maintained and the strong polyhedrin promoter drives high levels of cDNA transcription. Recombinant baculovirus is used to infect Spodoptera frugiperda (Sf9) insect cells in most cases, or human hepatocytes, in some cases. Infection of the latter requires additional genetic modifications to baculovirus. (See Engelhard, E.K. et al. (1994) Proc. Natl. Acad. Sci. USA 91:3224-3227; Sandig, V. et al. (1996) Hum. Gene Ther. 7:1937-1945.)

In most expression systems, PROAP is synthesized as a fusion protein with, e.g., glutathione S-transferase (GST) or a peptide epitope tag, such as FLAG or 6-His, permitting rapid, single-step, affinity-based purification of recombinant fusion protein from crude cell lysates. GST, a 26-kilodalton enzyme from Schistosoma japonicum, enables the purification of fusion proteins on immobilized glutathione under conditions that maintain protein activity and antigenicity (Amersham Pharmacia Biotech). Following purification, the GST moiety can be proteolytically cleaved from

PROAP at specifically engineered sites. FLAG, an 8-amino acid peptide, enables immunoaffinity purification using commercially available monoclonal and polyclonal anti-FLAG antibodies (Eastman Kodak). 6-His, a stretch of six consecutive histidine residues, enables purification on metal-chelate resins (QIAGEN). Methods for protein expression and purification are discussed in Ausubel (1995, supra, ch. 10 and 16). Purified PROAP obtained by these methods can be used directly in the following activity assay.

X. Demonstration of PROAP Activity

An assay for PROAP activity measures cell proliferation as the amount of newly initiated DNA synthesis in Swiss mouse 3T3 cells. A plasmid containing polynucleotides encoding PROAP is transfected into quiescent 3T3 cultured cells using methods well known in the art. The transiently transfected cells are then incubated in the presence of [³H]thymidine, a radioactive DNA precursor. Where applicable, varying amounts of PROAP ligand are added to the transfected cells. Incorporation of [³H]thymidine into acid-precipitable DNA is measured over an appropriate time interval, and the amount incorporated is directly proportional to the amount of newly synthesized DNA.

An alternative assay for PROAP activity measures the induction of apoptosis when PROAP is expressed at physiologically elevated levels in mammalian cell culture systems. cDNA is subcloned into a mammalian expression vector containing a strong promoter that drives high levels of cDNA expression. Vectors of choice include pCMV SPORT (Life Technologies, Gaithersburg, MD) and pCR 3.1 (Invitrogen, Carlsbad, CA, both of which contain the cytomegalovirus promoter. 5-10 μ g of recombinant vector are transiently transfected into a human cell line, preferably of endothelial or hematopoietic origin, using either liposome formulations or electroporation. 1-2 μ g of an additional plasmid containing sequences encoding a marker protein are co-transfected. Expression of a marker protein provides a means to distinguish transfected cells from nontransfected cells and is a reliable predictor of cDNA expression from the recombinant vector. Marker proteins of choice include, e.g., Green Fluorescent Protein (GFP) (Clontech, Palo Alto, CA), CD64, or a CD64-GFP fusion protein. Flow cytometry (FCM), an automated, laser optics-based technique, is used to identify transfected cells expressing GFP or CD64-GFP and to evaluate their apoptotic state. FCM detects and quantifies the uptake of fluorescent molecules that diagnose events preceding or coincident with cell death. These events include changes in nuclear DNA content as measured by staining of DNA with propidium iodide; changes in cell size and granularity as measured by forward light scatter and 90 degree side light scatter; down-regulation of DNA synthesis as measured by decrease in bromodeoxyuridine uptake; alterations in expression of cell surface and intracellular proteins as measured by reactivity with specific antibodies; and alterations in plasma membrane

composition as measured by the binding of fluorescein-conjugated Annexin V protein to the cell surface.

Alternatively, PROAP activity may be measured by the induction of growth arrest when PROAP is expressed at physiologically elevated levels in transformed mammalian cell lines. PROAP cDNA is subcloned into a mammalian expression vector containing a strong promoter that drives high levels of cDNA expression, and these constructs are stably transfected into a transformed cell line, such as NIH 3T6 or C6, using methods known in the art. An additional plasmid, containing sequences which encode a selectable marker, such as hygromycin resistance, are co-transfected. Expression of a marker protein provides a means to distinguish transfected cells from nontransfected cells and is a reliable predictor of cDNA expression from the recombinant vector. Cells expressing PROAP are compared with control cells, either non-transfected or transfected with vector alone, for characteristics associated with growth arrest. Such characteristics can include, but are not limited to, a reduction in [³H]-thymidine incorporation into newly synthesized DNA, lower doubling and generation times, and decreased culture saturation density.

Alternatively, an assay for PROAP activity uses radiolabeled nucleotides, such as [³²P]ATP, to measure either the incorporation of radiolabel into DNA during DNA synthesis, or fragmentation of DNA that accompanies apoptosis. Mammalian cells are transfected with plasmid containing cDNA encoding PROAP by methods well known in the art. Cells are then incubated with radiolabeled nucleotide for various lengths of time. Chromosomal DNA is collected, and radioactivity detected using a scintillation counter. Incorporation of radiolabel into chromosomal DNA is proportional to the degree of stimulation of the cell cycle. To determine if PROAP promotes apoptosis, chromosomal DNA is collected as above, and analyzed using polyacrylamide gel electrophoresis, by methods well known in the art. Fragmentation of DNA is quantified by comparison to untransfected control cells, and is proportional to the apoptotic activity of PROAP.

XI. Functional Assays

PROAP function is assessed by expressing the sequences encoding PROAP at physiologically elevated levels in mammalian cell culture systems. cDNA is subcloned into a mammalian expression vector containing a strong promoter that drives high levels of cDNA expression. Vectors of choice include pCMV SPORT (Life Technologies) and pCR3.1 (Invitrogen, Carlsbad CA), both of which contain the cytomegalovirus promoter. 5-10 μ g of recombinant vector are transiently transfected into a human cell line, for example, an endothelial or hematopoietic cell line, using either liposome formulations or electroporation. 1-2 μ g of an additional plasmid containing sequences encoding a marker protein are co-transfected. Expression of a marker protein

provides a means to distinguish transfected cells from nontransfected cells and is a reliable predictor of cDNA expression from the recombinant vector. Marker proteins of choice include, e.g., Green Fluorescent Protein (GFP; Clontech), CD64, or a CD64-GFP fusion protein. Flow cytometry (FCM), an automated, laser optics-based technique, is used to identify transfected cells expressing GFP or CD64-GFP and to evaluate the apoptotic state of the cells and other cellular properties. FCM detects and quantifies the uptake of fluorescent molecules that diagnose events preceding or coincident with cell death. These events include changes in nuclear DNA content as measured by staining of DNA with propidium iodide; changes in cell size and granularity as measured by forward light scatter and 90 degree side light scatter; down-regulation of DNA synthesis as measured by decrease in bromodeoxyuridine uptake; alterations in expression of cell surface and intracellular proteins as measured by reactivity with specific antibodies; and alterations in plasma membrane composition as measured by the binding of fluorescein-conjugated Annexin V protein to the cell surface. Methods in flow cytometry are discussed in Ormerod, M.G. (1994) Flow Cytometry, Oxford, New York NY.

The influence of PROAP on gene expression can be assessed using highly purified populations of cells transfected with sequences encoding PROAP and either CD64 or CD64-GFP. CD64 and CD64-GFP are expressed on the surface of transfected cells and bind to conserved regions of human immunoglobulin G (IgG). Transfected cells are efficiently separated from nontransfected cells using magnetic beads coated with either human IgG or antibody against CD64 (DYNAL, Lake Success NY). mRNA can be purified from the cells using methods well known by those of skill in the art. Expression of mRNA encoding PROAP and other genes of interest can be analyzed by northern analysis or microarray techniques.

XII. Production of PROAP Specific Antibodies

PROAP substantially purified using polyacrylamide gel electrophoresis (PAGE; see, e.g., Harrington, M.G. (1990) *Methods Enzymol.* 182:488-495), or other purification techniques, is used to immunize rabbits and to produce antibodies using standard protocols.

Alternatively, the PROAP amino acid sequence is analyzed using LASERGENE software (DNASTAR) to determine regions of high immunogenicity, and a corresponding oligopeptide is synthesized and used to raise antibodies by means known to those of skill in the art. Methods for selection of appropriate epitopes, such as those near the C-terminus or in hydrophilic regions are well described in the art. (See, e.g., Ausubel, 1995, supra, ch. 11.)

Typically, oligopeptides of about 15 residues in length are synthesized using an ABI 431A peptide synthesizer (Perkin-Elmer) using fmoc-chemistry and coupled to KLH (Sigma-Aldrich, St. Louis MO) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) to increase immunogenicity. (See, e.g., Ausubel, 1995, supra.) Rabbits are immunized with the oligopeptide-

KLH complex in complete Freund's adjuvant. Resulting antisera are tested for antipeptide and anti-PROAP activity by, for example, binding the peptide or PROAP to a substrate, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radio-iodinated goat anti-rabbit IgG.

XIII. Purification of Naturally Occurring PROAP Using Specific Antibodies

5 Naturally occurring or recombinant PROAP is substantially purified by immunoaffinity chromatography using antibodies specific for PROAP. An immunoaffinity column is constructed by covalently coupling anti-PROAP antibody to an activated chromatographic resin, such as CNBr-activated SEPHAROSE (Amersham Pharmacia Biotech). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

10 Media containing PROAP are passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of PROAP (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/PROAP binding (e.g., a buffer of pH 2 to pH 3, or a high concentration of a chaotrope, such as urea or thiocyanate ion), and PROAP is collected.

XIV. Identification of Molecules Which Interact with PROAP

15 PROAP, or biologically active fragments thereof, are labeled with ¹²⁵I Bolton-Hunter reagent. (See, e.g., Bolton A.E. and W.M. Hunter (1973) Biochem. J. 133:529-539.) Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled PROAP, washed, and any wells with labeled PROAP complex are assayed. Data obtained using
20 different concentrations of PROAP are used to calculate values for the number, affinity, and association of PROAP with the candidate molecules.

Various modifications and variations of the described methods and systems of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with certain embodiments, it should be
25 understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Table 1

Polypeptide SEQ ID NO:	Nucleotide SEQ ID NO:	Clone ID	Library	Fragments
1	20	1342011	COLNTUT03	1291596H1 (BRAINOT11), 485081X18 (HNT2RAT01), 671427H1 (CRBLNOT01), 1352964T6 (LATRTUT02), 1342011H1 (COLNTUT03), 1444182R1 (THRYNOT03), 1444182F1 (THRYNOT03)
2	21	1880041	LEUKNOT03	3470287H1 (BRAIDIT01), 1832158R6 (BRAINON01), 2288712H1 (BRAINON01), 1384536F1 (BRAITUT08), 1880041H1 (LEUKNOT03)
3	22	3201881	PENCNOT02	3201881H1 (PENCNOT02), 2520087F6 (BRAITUT21), 352438X15 (LVENNOT01)
4	23	939000	CERVNOT01	110900F1 (PITUNOT01), 548840F1 (BEPINOT01), 939000H1 (CERVNOT01), 939000X12 (CERVNOT01), 1271295F6 (TESTTUT02), 2122589F6 (BRSTNOT07), 3618041H1 (EPIPNOT01), SXAA02479D1, SXAA01641D1, SXAA01631D1, SAAO02385F1
5	24	2125677	BRSTNOT07	368085R1 (SYNORAT01), 392816H1 (TMLR2DT01), 518806R6 (MMLR1DT01), 1271911H1 (TESTTUT02), 1822315X314D1 (GBLATUT01), 1858290F6 (PROSNOT18), 2125677H1 (BRSTNOT07), 2293815H1 (BRAINON01), 2573443R6 (HIPOAZT01), 2764062H1 (BRSTNOT12), 2832044T6 (TLYMNOT03), 3428001H1 (BRSTNOR01), 3687264H1 (HEAANOT01), 3765525H1 (BRSTNOT24), 4590195H1 (MASTTUT01)
6	25	2603810	LUNGTUT07	013535R1 (THP1PLB01), 267329R1 (HNT2NOT01), 1453513F1 (PENITUT01), 1556582F6 (BLADTUT04), 2603810H1 (LUNGTUT07)
7	26	2715761	THYRNOT09	2715761H1 (THYRNOT09), 2993353F6 (KIDNFET02), SBLA03719F1

Table 1 (cont.)

Polypeptide SEQ ID NO:	Nucleotide SEQ ID NO:	Clone ID	Library	Fragments
8	27	3255641	OVARTUN01	516590H1 (MMLR1DT01), 1921460R6 (BRSTTUT01), 2824323F6 (ADRETUT06), 3255641H1 (OVARTUN01), 3255641R6 (OVARTUN01), SBXA03995D1
9	28	3620391	MENTNOT01	1556171H1 (BLADTUT04), 3620391H1 (MENTNOT01)
10	29	3969860	PROSTUT10	3969860H1 (PROSTUT10), 4275630F6 (PROSTMT01), 4275630T6 (PROSTMT01), 4403647F6 (PROSDIT01)
11	30	4286006	LIVRDIR01	4286006F6 (LIVRDIR01), 4286006H1 (LIVRDIR01)
12	31	4325626	TLYMUNT01	841543R1 (PROSTUT05), 841543X53 (PROSTUT05), 1752767F6 (LIVRTUT01), 2994209T6 (KIDNFET02), 3053308H1 (LNODNOT08), 4325626H1 (TLYMUNT01), 5209052H1 (BRAFNOT02)
13	32	1438978	PANCNOT08	834140H1 (PROSNOT07), 1438978F6 (PANCNOT08), 4074639H1 (PANCNOT19)
14	33	2024773	KERANOT02	782716R1 (MYOMNOT01), 980866R1 (TONGTUT), 1995464T6 (BRSTTUT03), 2027443H1 (KERANOT02), 2106331R6 (BRAITUT03), 3333150H1 (BRAIFET01)
15	34	3869790	BMARNOT03	359792R6 (SYNORAB01), 1535116T1 (SPLNNOT04), 2587946F6 (BRAITUT22), 3869790H1 (BMARNOT03)
16	35	001273	U937NOT01	001273H1 (U937NOT01), 1528039F1 (UCMCL5T01), 1526245F6 (UCMCL5T01), 899008R6 (BRSTTUT03), 022308F1 (ADENINB01)

Table 1 (cont.)

Polypeptide SEQ ID NO:	Nucleotide SEQ ID NO:	Clone ID	Library	Fragments
17	36	411831	BRSTNOT01	411831H1 (BRSTNOT01), 1232212F1 (LUNGFET03), 1997123R6 (BRSTTUT03), 001732H1 (U937NOT01), 414405T6 (BRSTNOT01), 781412R1 (MYOMNOT01), SADC11822F1
18	37	1520835	BLADTUT04	1419118F6 (KIDNNOT09), 1520835F1 and 1520835H1 (BLADTUT04), 1529102F6 (UCMCL5T01), 3842242F6 (DENDNOT01)
19	38	1902803	OVARNOT07	180897F1 (PLACNOB01), 491345H1 (HNT2AGT01), 927993R1 (BRAINOT04), 1902803H1 (OVARNOT07), 4217475H1 (ADRENOT15)

Table 2

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Identification	Analytical Methods
1	334	S122 T60 S192 S203 S204 S218 S89 S118 S226	N190		Mouse npdcf-1 (g452276)	BLAST
2	281	S120 S44 S180 S245 S284 S285 T295 S143 T225 T232			Human EB1 (g998357)	BLAST
3	237	S16 T33 S149 S172 S190 Y119	N14 N25 N31 N147		Mouse serum deprivation response protein (sdr) (g455719)	BLAST
4	941	T542 T858 T30 T55 T76 T153 S159 T198 T249 T266 S300 T432 S653 S750 T29 S315 T322 T357 S372 S403 T462 S493 S572 T674 S681 S783 S853 T867 Y131 Y658	N74 N196		TPR protein (Zer1p) (g1209391)	MOTIFS BLAST

Table 2 (cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Identification	Analytical Methods
5	918	T19 T94 S469 T2 S44 T82 S107 T120 S257 T276 T399 S475 S579 S605 S708 S715 S785 T790 S814 S835 S841 S8 S22 S29 S60 S198 S251 S285 T374 S556 S589 S602 T634 S697 T843 T872 S897	N116	Polyadenylate binding (PABP) protein domain: P87-D126 F139-G185 R492-I568 HECT (ubiquitin transferase) domain: S605-V918	Drosophila hyper-plastic discs (HYD) protein (g2673887)	MOTIFS BLAST PFAM BLOCKS
6	324	S140 S191 S273 T287 S226		Mitochondrial energy transfer protein signature: P141-L149 Transmembrane domains: V306-I324 A33-R53	Similar to human growth arrest inducible gene product (g1707054)	MOTIFS BLAST HMM
7	185	T72 T73 T132 T21 T160 T174 S35 S95			APC10 (Anaphase promoting complex) (g3402334)	MOTIFS BLAST

Table 2 (cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Identification	Analytical Methods
8	445	T281 S32 S118 S135 S177 S416 T418 T81 T186 T203 S262 S302 T335 T346	N300 N414	Rhodopsin-like GPCR fingerprint: F282-L306 Transmembrane domains: I147-Y166 S357-Y373	Mitogen-induced protein (g2290726)	MOTIFS BLAST PRINTS HMM
9	73	T55 T15 S25 S28 T50	N34		Cyclin E (g1262821)	MOTIFS BLAST
10	288	T159 T161 S190 S228 S245 S56 S117 S120 S143 S190 T240	N226	SPRY domain: E132-W153 C148-M273 C3HC4 zinc finger: C11-Q39	RET finger protein-like 1, long variant (g3417312)	MOTIFS BLAST PFAM BLOCKS
11	98	T61 S22 Y57 Y69 Y90	N59	SH3 domain: A46-E64	Melanoma inhibitor protein homolog (g1778171)	MOTIFS BLAST BLOCKS PRINTS

Table 2 (cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Identification	Analytical Methods
12	549	S139 T313 T351 T61 T460 S484 T511 S73 S90 S91 T152 S216 T282 T315 S346 S446 Y99		Probable rabGAP domain: A98-T315	TRE oncogene product (g37330)	MOTIFS BLAST PFAM
13	95	T9 S10 S20 T48			Human dimlp homolog (g2565275)	BLAST
14	445	T14 T24 T109 S142 T213 T244 S275 Y297 S300 S355 S361 S372 S393 T425 T432	N269 N284 N370		Fly FAS-associated factor (FFAF) (g3688609)	BLAST
15	219	T46 T55 T82 T199	N18		Cell death activator CIDE-B (g3114594)	BLAST
16	439	T27 T32 S75 S123 S347 T381 T404 T263 Y231 Y294		Signal peptide: M1-A28	p52 apoptotic protein (g259942)	MOTIFS BLAST HMM

Table 2 (cont.)

Polypeptide SEQ ID NO:	Amino Acid Residues	Potential Phosphorylation Sites	Potential Glycosylation Sites	Signature Sequence	Identification	Analytical Methods
17	526	S383 S470 S69 S78 S137 T273 T274 S342 S432 T453 S231 T285 T290 S342 T360 T407 S423 S436 S460 S508	N217 N229	bZIP transcription factor: K384-R398 Cyclin cell cycle division protein: A224-I250 Signal peptide: M1-S25	cyclin ania-6a g5453421 [Mus musculus]	MOTIFS BLAST BLOCKS HMM
18	298	T63 S93 S165 S212 S220 S6 T44 S133 T203 T251		C3HC4 type Zn finger: C267-A276 apoptosis inhibitor: R90-L155	putative apoptosis inhibitor (g2957175)	MOTIFS PFAM PROFILESAN BLAST
19	249	S57 S119 T134 S150 T167 S205 S52 S125 T230 Y121		PHD finger: P196-E245	candidate tumor suppressor (g2829208)	MOTIFS BLAST PFAM

Table 3

Polynucleotide SEQ ID NO:	Selected Fragments	Tissue Expression (Fraction of Total)	Disease or Condition (Fraction of Total)	Vector
20	518-568		Cell Proliferation (0.660) Inflammation/Trauma (0.270)	pINCY
21	613-693		Cell Proliferation (0.560)	pINCY
22	949-984		Cell Proliferation (0.560)	pINCY
23	811-855 1297-1341	Reproductive (0.287) Nervous (0.181) Hematopoietic/Immune (0.138)	Cancer (0.487) Inflammation (0.250) Cell Proliferation (0.181)	PSPORT1
24	275-322 1955-1999	Reproductive (0.279) Nervous (0.174) Hematopoietic/Immune (0.116)	Cancer (0.419) Inflammation (0.267) Cell Proliferation (0.174)	pINCY
25	322-351	Reproductive (0.306) Cardiovascular (0.105) Hematopoietic/Immune (0.105)	Cancer (0.484) Inflammation (0.290) Cell Proliferation (0.234)	pINCY
26	658-702	Reproductive (0.444) Developmental (0.111) Hematopoietic/Immune (0.111)	Cancer (0.500) Inflammation (0.333) Cell Proliferation (0.167)	pINCY
27	172-216 604-648	Reproductive (0.256) Nervous (0.186) Hematopoietic/Immune (0.163)	Cancer (0.349) Inflammation (0.302) Trauma (0.116)	PSPORT1
28	58-102	Musculoskeletal (1.000)	Cancer (1.000)	pINCY

Table 3 (cont.)

Polynucleotide SEQ ID NO:	Selected Fragments	Tissue Expression (Fraction of Total)	Disease or Condition (Fraction of Total)	Vector
29	217-246 433-477	Reproductive (0.455) Nervous (0.273) Cardiovascular (0.091)	Cancer (0.455) Cell Proliferation (0.182) Trauma (0.182)	pINCY
30	257-301	Gastrointestinal (1.000)	Inflammation (1.000)	pINCY
31	219-263 1569-1613	Gastrointestinal (0.245) Nervous (0.245) Reproductive (0.245)	Cancer (0.490) Inflammation (0.265) Cell Proliferation (0.143)	pINCY
32	585-629	Nervous (0.390) Reproductive (0.150)	Cancer and Cell Proliferation (0.690)	
33	381-425	Reproductive (0.310) Nervous (0.150)	Cancer and Cell Proliferation (0.650)	
34	133-177	Reproductive (0.330)	Cancer (0.440)	
35	110-154	Reproductive (0.282) Hematopoietic/Immune (0.256) Cardiovascular (0.154)	Cancer (0.462) Inflammation (0.256) Fetal (0.179)	PBLUESCRIPT
36	164-208	Reproductive (0.236) Gastrointestinal (0.181) Hematopoietic/Immune (0.153)	Cancer (0.486) Inflammation (0.264) Fetal (0.125)	PBLUESCRIPT

Table 3 (cont.)

Polynucleotide SEQ ID NO:	Selected Fragments	Tissue Expression (Fraction of Total)	Disease or Condition (Fraction of Total)	Vector
37	272-316	Developmental (0.429) Hematopoietic/Immune (0.286) Reproductive (0.143) Urologic (0.143)	Fetal (0.571) Cancer (0.286) Inflammation (0.143)	pINCY
38	782-826	Reproductive (0.253) Nervous (0.176) Urologic (0.121)	Cancer (0.440) Inflammation (0.242) Fetal (0.231)	pINCY

Table 4

Polynucleotide SEQ ID NO:	Library	Library Comment
20	COLNTUT03	This library was constructed using RNA isolated from colon tumor tissue obtained from the sigmoid colon of a 62-year-old Caucasian male during a sigmoidectomy and permanent colostomy. Pathology indicated invasive grade 2 adenocarcinoma. One lymph node contained metastasis with extranodal extension. Patient history included hyperlipidemia, cataract disorder, and dermatitis. Family history included benign hypertension, atherosclerotic coronary artery disease, hyperlipidemia, breast cancer, and prostate cancer.
21	LEUKNOT03	This library was constructed using RNA isolated from white blood cells of a 27-year-old female with blood type A+. The donor tested negative for cytomegalovirus (CMV).
22	PENCNOT02	This library was constructed using RNA isolated from penis right corpus cavernosum tissue.
23	CERVNOT01	This library was constructed using RNA isolated from uterine cervical tissue of a 35-year-old Caucasian female during a vaginal hysterectomy with dilation and curettage. Pathology indicated mild chronic cervicitis. Family history included atherosclerotic coronary artery disease and type II diabetes.
24	BRSTNOT07	This library was constructed using RNA isolated from diseased breast tissue removed from a 43-year-old Caucasian female during a unilateral extended simple mastectomy. Pathology indicated mildly proliferative fibrocystic changes with epithelial hyperplasia, papillomatosis, and duct ectasia. Pathology for the associated tumor tissue indicated invasive grade 4, nuclear grade 3 mammary adenocarcinoma with extensive comedo necrosis. Family history included epilepsy, cardiovascular disease, and type II diabetes.

Table 4 (cont.)

Polynucleotide SEQ ID NO:	Library	Library Comment
25	LUNG-TUT07	This library was constructed using RNA isolated from lung tumor tissue removed from the upper lobe of a 50-year-old Caucasian male during segmental lung resection. Pathology indicated an invasive grade 4 squamous cell adenocarcinoma. Patient history included tobacco use. Family history included skin cancer.
26	THYRNOT09	This library was constructed using RNA isolated from diseased thyroid tissue removed from an 18-year-old Caucasian female during a unilateral thyroid lobectomy and regional lymph node excision. Pathology indicated adenomatous goiter associated with a follicular adenoma of the thyroid. Family history included thyroid cancer.
27	OVARTUN01	This normalized library was constructed from 5.36 million independent clones obtained from an ovarian tumor library. RNA was isolated from tumor tissue removed from the left ovary of a 58-year-old Caucasian female during a total abdominal hysterectomy, removal of a single ovary, and inguinal hernia repair. Pathology indicated metastatic grade 3 adenocarcinoma of colonic origin, forming a partially cystic and necrotic tumor mass in the left ovary and a nodule in the left mesovarium. A single intramural leiomyoma was identified in the myometrium. The cervix showed mild chronic cystic cervicitis. Patient history included benign hypertension, follicular ovarian cyst, colon cancer, benign colon neoplasm, and osteoarthritis. Family history included emphysema, myocardial infarction, atherosclerotic coronary artery disease, benign hypertension, hyperlipidemia, and primary tuberculous complex. The normalization and hybridization conditions were adapted from Soares et al. (PNAS (1994) 91:9928) and Bonaldo et al. (Genome Research (1996) 6:791).

Table 4 (cont.)

Polynucleotide SEQ ID NO:	Library	Library Comment
28	MENTNOT01	This library was constructed using RNA isolated from left tibial meniscus tissue removed from a 16-year-old Caucasian male during a partial left tibial osteotomy with free skin graft. Pathology for the associated tumor indicated metastatic alveolar rhabdomyosarcoma. Patient history included an abnormality of the red blood cells. Family history included osteoarthritis.
29	PROSTUT10	This library was constructed using RNA isolated from prostatic tumor tissue removed from a 66-year-old Caucasian male during radical prostatectomy and regional lymph node excision. Pathology indicated an adenocarcinoma (Gleason grade 2+3) and adenofibromatous hyperplasia. The patient presented with elevated prostate specific antigen (PSA). Family history included prostate cancer and secondary bone cancer.
30	LIVRDIR01	This library was constructed using RNA isolated from diseased liver tissue removed from a 63-year-old Caucasian female during a liver transplant. Patient history included primary biliary cirrhosis. Serology was positive for anti-mitochondrial antibody.
31	TYLMUNT01	This library was constructed using RNA isolated from resting allogenic T-lymphocyte tissue removed from an adult (40-50-year-old) Caucasian male.
32	PANCNOT08	This library was constructed using RNA isolated from pancreatic tissue removed from a 65-year-old Caucasian female during radical subtotal pancreatectomy. Pathology for the associated tumor tissue indicated an invasive grade 2 adenocarcinoma. Patient history included type II diabetes, osteoarthritis, cardiovascular disease, benign neoplasm in the large bowel, and a cataract.
33	KERANOT02	This library was constructed using RNA isolated from epidermal breast keratinocytes (NHEK). NHEK (Clontech #CC-2501) is human breast keratinocyte cell line derived from a 30-year-old black female during breast-reduction surgery.

Table 4 (cont.)

Polynucleotide SEQ ID NO:	Library	Library Comment
34	BMARNOT03	This library was constructed using RNA isolated from the left tibial bone marrow tissue of a 16-year-old Caucasian male during a partial left tibial osteotomy with free skin graft. Patient history included an abnormality of the red blood cells. Previous surgeries included bone and bone marrow biopsy, and soft tissue excision.
35	U937NOT01	This library was constructed at Stratagene (STR937207), using RNA isolated from the U937 monocyte-like cell line. This line (ATCC CRL1593) was established from malignant cells obtained from the pleural effusion of a 37-year-old Caucasian male with diffuse histiocytic lymphoma.
36	BRSTNOT01	This library was constructed using RNA isolated from the breast tissue of a 56-year-old Caucasian female who died in a motor vehicle accident.
37	BLADTUT04	This library was constructed using RNA isolated from bladder tumor tissue removed from a 60-year-old Caucasian male during a radical cystectomy, prostatectomy, and vasectomy. Pathology indicated grade 3 transitional cell carcinoma in the left bladder wall. Carcinoma in-situ was identified in the dome and trigone. Family history included type I diabetes, a malignant neoplasm of the stomach, atherosclerotic coronary artery disease, and an acute myocardial infarction.
38	OVARNOT07	This library was constructed using RNA isolated from left ovarian tissue removed from a 28-year-old Caucasian female during a vaginal hysterectomy and removal of the fallopian tubes and ovaries. The tissue was associated with multiple follicular cysts, endometrium in a weakly proliferative phase, and chronic cervicitis of the cervix with squamous metaplasia. Family history included benign hypertension, hyperlipidemia, and atherosclerotic coronary artery disease.

Table 5

Program	Description	Reference	Parameter Threshold
ABI FACTURA	A program that removes vector sequences and masks ambiguous bases in nucleic acid sequences.	Perkin-Elmer Applied Biosystems, Foster City, CA.	
ABI/PARACEL FDF	A Fast Data Finder useful in comparing and annotating amino acid or nucleic acid sequences.	Perkin-Elmer Applied Biosystems, Foster City, CA; Paracel Inc., Pasadena, CA.	Mismatch <50%
ABI AutoAssembler	A program that assembles nucleic acid sequences.	Perkin-Elmer Applied Biosystems, Foster City, CA.	
BLAST	A Basic Local Alignment Search Tool useful in sequence similarity search for amino acid and nucleic acid sequences. BLAST includes five functions: blastp, blastn, blastx, tblastn, and tblastx.	Altschul, S.F. et al. (1990) J. Mol. Biol. 215:403-410; Altschul, S.F. et al. (1997) Nucleic Acids Res. 25: 3389-3402.	ESTs: Probability value= 1.0E-8 or less Full Length sequences: Probability value= 1.0E-10 or less
FASTA	A Pearson and Lipman algorithm that searches for similarity between a query sequence and a group of sequences of the same type. FASTA comprises at least five functions: fasta, tfasta, fastx, tfastx, and ssearch.	Pearson, W.R. and D.J. Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-2448; Pearson, W.R. (1990) Methods Enzymol. 183: 63-98; and Smith, T.F. and M. S. Waterman (1981) Adv. Appl. Math. 2:482-489.	ESTs: fasta E value=1.06E-6 Assembled ESTs: fasta Identity= 95% or greater and Match length=200 bases or greater; fastx E value=1.0E-8 or less Full Length sequences: fastx score=100 or greater
BLIMPS	A BLocks IMProved Searcher that matches a sequence against those in BLOCKS and PRINTS databases to search for gene families, sequence homology, and structural fingerprint regions.	Henikoff, S and J.G. Henikoff, Nucl. Acid Res., 19:6565-72, 1991. J.G. Henikoff and S. Henikoff (1996) Methods Enzymol. 266:88-105; and Attwood, T.K. et al. (1997) J. Chem. Inf. Comput. Sci. 37: 417-424.	Score=1000 or greater; Ratio of Score/Strength = 0.75 or larger; and Probability value= 1.0E-3 or less
PFAM	A Hidden Markov Models-based application useful for protein family search.	Krogh, A. et al. (1994) J. Mol. Biol., 235:1501-1531; Sonnhammer, E.L.L. et al. (1988) Nucleic Acids Res. 26:320-322.	Score=10-50 bits, depending on individual protein families

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Table 5 cont.

Program	Description	Reference	Parameter Threshold
ProfileScan	An algorithm that searches for structural and sequence motifs in protein sequences that match sequence patterns defined in Prosite.	Gribskov, M. et al. (1988) CABIOS 4:61-66; Gribskov, et al. (1989) Methods Enzymol. 183:146-159; Bairoch, A. et al. (1997) Nucleic Acids Res. 25: 217-221.	Score= 4.0 or greater
Phred	A base-calling algorithm that examines automated sequencer traces with high sensitivity and probability.	Ewing, B. et al. (1998) Genome Res. 8:175-185; Ewing, B. and P. Green (1998) Genome Res. 8:186-194.	
Phrap	A Phils Revised Assembly Program including SWAT and CrossMatch, programs based on efficient implementation of the Smith-Waterman algorithm, useful in searching sequence homology and assembling DNA sequences.	Smith, T.F. and M. S. Waterman (1981) Adv. Appl. Math. 2:482-489; Smith, T.F. and M. S. Waterman (1981) J. Mol. Biol. 147:195-197; and Green, P., University of Washington, Seattle, WA.	Score= 120 or greater; Match length= 56 or greater
Consed	A graphical tool for viewing and editing Phrap assemblies	Gordon, D. et al. (1998) Genome Res. 8:195-202.	
SPScan	A weight matrix analysis program that scans protein sequences for the presence of secretory signal peptides.	Nielson, H. et al. (1997) Protein Engineering 10:1-6; Claverie, J.M. and S. Audic (1997) CABIOS 12: 431-439.	Score=5 or greater
Motifs	A program that searches amino acid sequences for patterns that matched those defined in Prosite.	Bairoch et al. <u>supra</u> ; Wisconsin Package Program Manual, version 9, page M51-59, Genetics Computer Group, Madison, WI.	